The dispersal, survival and genetic variability within *Dichelobacter nodosus* strains, the causal agent of ovine foot disease

MOHD MUZAFAR

BVSc, MVSc (Hons)

A thesis submitted for the degree of Doctor of Philosophy

School of Life Sciences

University of Warwick

February 2016

CONTENTS

LIST OF ABBREVIATIONS

Ac	Reverse primer for *D. nodosus* 16S rRNA
AL Buffer	Lysis Buffer
AW 1	Wash Buffer 1
AW2	Wash Buffer 2
AE	Elution Buffer
BBQ	Black Berry Quencher
BE	Blank Extraction
BH	Bedding Hoof
BLAST	Basic Local Alignment Software Tool
bp	base pair
BSA	Bovine Serum Albumin
BS	Straw Bedding
BS4	Benign strain of D. nodosus from the UK
B5	Wash Buffer
C	Coincidence index of overlap
Cc	Forward primer for D. nodosus 16S rRNA
cDNA	Complementary Deoxyribonucleic Acid
CODD	Contagious Ovine Interdigital Dermatitis
D	Time interval in days for rate calculated
DAPI	4',6-diamidino-2-phenylindole
DES	DNase Free Water
DNA	Deoxyribonucleic Acid
DNTR	*Dichelobacter nodosus* Tandem Repeat
E	Occurrence of MLVA alleles in ewes
E1	Ewe1
EB	Elution Buffer

FAM	6-Carboxy-fluorescein
H	Healthy
HH	Hoof Horn
ID	Interdigital Dermatitis
kb	Kilo Bases
kPa	Kilo Pascals
L	Occurrence of MLVA alleles in lambs
L1	Lamb1
LB	Luria Bertani Broth/Agar
LIZ	Genescan 1200 Liz size standard
LF	Left Front foot
LR	Left Hind/Rare foot
MHC	Major Histocompatibility Complex
MLVA	Multi Locus Variable Number Tandem Repeat Analysis
MP	Matric Potential
mRNA	Messenger RNA
NGS	Next Generation Sequencing
NH	Non Hoof Horn
NPMA	Non Propidium Monoazide
NTC	Non-template Control
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
PI	Propidium Iodide
PMA	Propidium Monoazide
pgr	Proline glycine repeat gene
qPCR	Quantitative Polymerase Chain Reaction
RF	Right front foot
RR	Right Hind/Rare foot
rpoD	RNA polymerase sigma 70 factor

rRNA	Ribosomal Ribonucleic acid
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
TAE	Tris base, acetic acid and EDTA (buffer)
TAS	Trypticase Arginine Serine
TASH	Trypticase Arginine Serine Hoof Agar
t_d	Death Rate
TOC	Total Organic carbon
UV	Ultraviolet light
VFR	Virulent Footrot
VCS1703A	Virulent strain of *D. nodosus* (Type strain).
WGS	Whole Genome Sequencing

ACKNOWLEDGEMENTS

All praise and thanks to The Almighty ALLAH who has always guided me towards the right path.

I would like to thank my supervisors Professor Elizabeth Margaret Helen Wellington and Professor Laura Elizabeth Green for giving me the opportunity to pursue PhD at Warwick University and for their encouragement, support, guidance and help throughout my years at Warwick. I consider myself lucky to have you both as my supervisor. My stay at Warwick has been an amazing experience which I will never forget. I am highly thankful to Prof. Wellington for allowing me to present my work at a number of national and international conferences. I am grateful to Warwick University for the Chancellor's International Scholarship.

I would also like to thank all members of C123 Wellington group especially Dr. Leo Calvo-Bado, Hayley and Dr. Edward Smith from Green group for their help.

I am grateful to Prof. Julian Rood from Monash University for his willingness to have me visiting his lab. I thank Dr. Ruth Kennan and Dr. Deiter Bulach for their cooperation and help during my visit to Monash University, Australia. I would like to extend my thanks to Dr. Rose Grogono-Thomas from Bristol University for her cooperation and for providing me the isolates and necessary information to complete this thesis. I am grateful to Dr. Dieter Bulach from Monash University for his help with Bioinformatics and Dr. Andy Millard for sequencing *D. nodosus* isolates.

A special thank you to Sheema for her unconditional love and support during the years of my PhD and for always being there. I express thanks to my friends Qaiser, Shameem and Arsheed for their support and encouragement.

I want to thank all my family members especially my grandmother Tathi, Raj Baya, Bilal Sahib, Mujeeb, Mouzoon Didi, Shani, Deeba Didi, Manzoor Uncle, Mushtaq Uncle, Dr Shah Junaid Rafique, Benazir Khan, Mushbiq, Muntaha, Musa, Essa, Mehwish, Shabia, Sana and Nayrah for their sustained faith in me and for their love and encouragement. Dr Shah Junaid Rafique and Benazir Khan deserve special thanks for their constructive criticism and for always being there whenever I needed them.

A big thanks to my father Nisar Ahmad Makhdoomi (Abbu Ji) who sadly passed away, for his love and continued advice. I dedicate this thesis to my dad.

DECLARATION

I declare that the results presented in this thesis were conducted by me under the supervision of Professor Elizabeth Wellington and Professor Laura Green. This is an original work which has not been submitted elsewhere for the award of any degree. The work was conducted in accordance with the local, national and international regulations and scientific ethical principles. All other sources of information used in this thesis has been specifically acknowledged by a reference to that particular source.

LIST OF FIGURES

LIST OF TABLES

AWARD/SCHOLARSHIPS RECEIVED DURING THE Ph.D

- Recipient of Warwick University Award, Chancellor's International Scholarship/School of Life Sciences - 1150257, 2011 – 2015.

- Recipient of the best talk award and student scholarship at the Postgraduate Symposium, School of Life Sciences, University of Warwick 2014.

- Recipient of Warwick Monash Alliance Seed Fund to conduct a research project at Monash University, Australia, 2013.

- Recipient of Society of General Microbiology President's Fund for Research Visit grants 2013.

- Recipient of the best poster award and a student scholarship at the Postgraduate Symposium, School of Life Sciences, University of Warwick 2013.

- Recipient of student scholarship at Vetpath 2012, 2nd Prato Conference on the Pathogenesis of Bacterial Diseases of Animals, Prato, Italy 2012

• LIST OF PUBLICATIONS

- The role of the environment in transmission of *Dichelobacter nodosus* between ewes and their lambs. **Muzafar M**, Calvo-Bado LA, Green LE, Smith ES, Russell CL, Grogono-Thomas, R, Wellington EMH. ***Veterinary Microbiology*** 179 (2015) 53–59.

- Survival of the ovine footrot pathogen *Dichelobacter nodosus* in different soils. **Muzafar M**, Green LE, Calvo-Bado LA, Tichauer E, King H, James P, Wellington EMH *Anaerobe* 38: 81-87.

- Genomic diversity and distribution of virulence determinants in UK isolates of *Dichelobacter nodosus.* **Muzafar M,** Green LE, Calvo-Bado LA, Smith E, Thomas RG, Wellington EMH (2015) **Manuscript in preparation.**

SUMMARY

Ovine footrot is an infectious disease of sheep which causes serious economic losses to countries worldwide and costs the UK £84 M per annum. For effective control of this disease which is a major animal welfare issue, it is essential to study mechanisms of transmission. One of the key aims of the project was to improve our understanding of the potential for *Dichelobacter nodosus,* the causal agent, transmission via the environment within a flock of ewes and lambs. Newborn lambs were free from *D. nodosus* presence on their feet, but rapidly became contaminated with *D. nodosus* within 5-13 h after birth as detected using the specific molecular markers *rpoD* and 16S rRNA gene. The likely source of contamination of lambs was the straw bedding from the communal pens. A diverse population of *D. nodosus* was observed on the feet of ewes and lambs as determined by the presence of multiple strains with variable numbers of *pgrA* tandem repeats in the R1 region. This was further supported by Multi Locus Variable Number Tandem Repeats (MLVA) typing of the isolates, which also indicated high variation in the alleles present on the ewes and lambs. The primary aim was to determine if multiple strains present on the ewes were vertically transmitted to the lambs. This work has clearly demonstrated that no vertical transmission occurred between ewes and lambs and some strains, but not all were shared between ewe and its lamb suggesting transmission from their mother or other ewes sharing the same lambing pen. *D. nodosus* was detected in a range of environmental samples such as bedding, faecal balls compacted within the interdigital space and soil suggesting that shedding into the environment is the main route of *D. nodosus* transmission. Survival studies provided evidence that the pathogen persisted in soil microcosms for at least 40 days with viable cells persisting for a minimum of 30 days in four soil types. A lower temperature of 5 ^{o}C and clay soil was associated with longer duration of survival. Single Nucleotide Polymorphism (SNP) analysis of isolates from the ewes and lambs indicated that two main clonal populations existed that represented two clusters α and β within clade I, a virulent clade from the recent genome study including 103 global *D. nodosus* strains. No UK strains grouped with the benign clade II. Previous diversity studies on the isolates indicated diversity in MLVA types, *pgr* alleles and *pgrA* tandem repeats, but these did not correlate with the clustering; clusters α and β contained a mix of *pgrA* and *pgrB* but both were in clade I. This conflicts with the role of *pgrB* as a non-virulent allele although *pgrA* expression was induced by hoof horn in vitro, whereas *pgrB* was not. The work reported in this thesis has improved our understanding of the environmental transmission of *D. nodosus* between sheep, longevity of the pathogen in soil and diversity of strains in the UK.

CHAPTER 1

General Introduction

1.1. Introduction to ovine footrot.

Ovine footrot is an infectious disease of sheep that is caused by the aero-tolerant anaerobic bacterium *Dichelobacter nodosus* (*D. nodosus*) formerly known as *Bacteroides nodosus* (Dewhirst et al., 1990; Moore, 2005), which is the essential transmitting agent (Beveridge, 1941). The disease is characterized by inflammation of the interdigital skin with or without separation of the hoof horn from the dermis. Both conditions result in lameness (Egerton et al., 1969; Kennan et al, 2011; Stewart et al., 1984). In the UK, footrot is the main cause of lameness in sheep (Kaler and Green, 2008) and a major animal welfare issue (Fitzpatrick et al., 2006). The prevalence of lameness in the UK has been reported in 95% flocks with a within-flock prevalence of 10% (Kaler and Green, 2008; Moore, 2005), and UK farmers have reported lameness in their flocks with either of the two clinical presentations, interdigital dermatitis (ID), or virulent footrot (VFR) (Wassink et al., 2010b). A large number of factors contribute to footrot in sheep, the most important being the presence of the pathogen and climatic conditions combined with host susceptibility e.g. poor foot integrity (Kaler et al., 2010b). Increasing rainfall and temperatures favour the spread of footrot in sheep (Smith et al., 2014), however, many farmers report the highest prevalence of footrot in ewes in winter, possibly because ewes are housed or left untreated (Wassink et al., 2003).

Ovine footrot was reported in Australia in 1941 (Beveridge, 1941). The disease is present world-wide, in the UK, New Zealand, USA, Canada, France, Spain, Germany, Norway, Sweden, Switzerland, Nepal, Bhutan, Malaysia and India (Belloy, 2007; Ghimire et al., 1996; Gilhuus et al., 2013; Gradin and Schmitz, 1977; Gurung, 2006;

Konig et al., 2011; M.R., 1991 ; Moore et al., 2005a; Naylor et al., 1998; Olson et al., 1998; Piriz Duran et al., 1990; Wani et al., 2007; Zakaria et al., 1998; Zhou and Hickford, 2000; Zhou et al., 2010).

Footrot has significant financial impact on the meat and wool industries. This is due to a reduced production, reduced wool strength and the costs related to treatment and prevention of disease (Egerton et al., 2004; Green and George, 2008; Stewart, 1989; Wani and Samanta, 2006). In some countries, this disease may also result in death of sheep due to starvation, thirst and systemic bacterial infections (Stewart, 1989); although this is not reported in the UK. Recent reports indicate that footrot in sheep results in annual losses of between £24 and £84 million in the UK and these are associated with treatment, control and prevention and production costs (Nieuwhof and Bishop, 2005; Wassink et al., 2010a). However, reports from Australia indicate the estimated cost to be $18.4 M in the year 2005-2006 (Sackett, 2006) and $42.6 million in New South Wales (Egerton et al., 2004).

Footrot has been reported to occur in sheep, goats, cattle, deer and pigs (Beveridge, 1941; Claxton et al., 1983; Piriz et al., 1996). Sheep and goats are the reservoir of infection and develop VFR, whereas cattle and deer develop less severe form of the disease. Soays (Bristish sheep breeds) are reported to be less susceptible to footrot whereas Merino sheep are highly susceptible (Emery et al., 1984). The disease occurs in cattle breeds such as Holstein-Friesian, Sahiwal, Brahman, Charolais cross that are highly susceptible (Frisch, 1976; Richards et al., 1980). It has been reported that *D*.

nodosus strains isolated from the cattle were unable to cause VFR in sheep. Therefore, cattle are not considered as the reservoir of infection for sheep and goats (Beveridge, 1941; Egerton and Laing, 1979). In cattle the disease appears as ID with small circular ulcers in the interdigital epithelium and severe cases are often characterized by ulceration, necrosis and under running of the hoof horn (Egerton and Laing, 1979; Richards et al., 1980). Footrot also occurs in pigs and is characterized by while line lesion around the heel known as "bush foot". It has been reported that the predominant prevalence of serogroup B was found in pigs (Piriz et al., 1996).

The disease is classified into two main clinical presentations viz ID and VFR and both conditions were regarded as two separate diseases (Winter, 2008) with ID being viewed by the UK farmers as non-infectious disease caused due to the environmental factors (Wassink et al., 2005). However, the recent literature suggests that ID and VFR are two different stages of the same disease (Calvo-Bado et al., 2011b; Moore et al., 2005b) and the load of *D. nodosus* is higher both before and during episodes of ID and VFR than on healthy feet (Witcomb et al., 2014). Conversely, Australian nomenclature classifies the disease into benign and virulent footrot (Abbott and Egerton, 2003) with benign and virulent forms corresponding to ID and VFR as per the UK classification (Green and George, 2008; Kennan et al., 2010).

1.1.1. Clinical presentation.

The clinical conditions of disease vary depending on how severe the lesions are, ability to cause under running of the hoof horn, number of sheep affected and proclivity for

self-cure (Liu, 1995). The disease severity varies from ID that does not develop further to VFR that causes severe damage of the hoof horn leading to separation of the hoof horn from the underlying soft tissue of the foot (Figure 1) (Beveridge, 1941; Moore et al., 2005b; Stewart, 1989). The under running starts at the junction of skin fold with the heel that spreads anteriorly to the abaxial wall and causes the hoof horn to separate from the foot laminae (Abbott and Lewis, 2005). This is accompanied by discharge of greyish exudate with the characteristic foetid odour (Beveridge, 1941). It has been suggested that the host's acute inflammatory response to *D. nodosus* infection may be responsible for the clinical signs to occur (Egerton et al., 1969). Conversely, benign cases are characterized by inflammation of the interdigital epithelium leading to mild lameness that heals quickly (Egerton et al., 1969; Glynn, 1993).

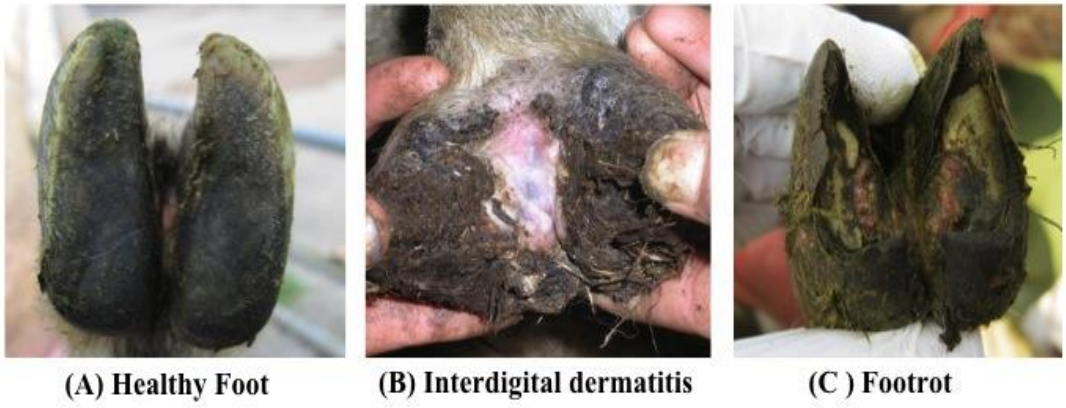


Figure 1.1. Clinical presentation of footrot in sheep. (A) Healthy foot with intact hoof and no signs of inflammation. (B) Foot with Interdigital dermatitis. Clear signs of inflammation of the interdigital skin (C) Foot with severe under running of the hoof horn and necrosis.

1.1.2. Etiology, pathogenesis, diagnosis, virulence tests, differential diagnosis and other hoof diseases.

The disease is caused by the synergistic action of several bacteria including *Dichelobacter nodosus* formerly known as *Fusiformis nodosus or Bacteroides nodosus* (Beveridge, 1941; Dewhirst et al., 1990; Moore et al., 2005a), *Fusobacterium necrophorum (F. necrophorum)*, *Trueperella pyogenes, Staphylococcus aureus, and Treponema* species (Collighan and Woodward, 1997; Dhawi et al., 2005; Egerton et al., 1969; Lavín, 2004; Naylor et al., 1998), of which *D. nodosus* is essential for causing footrot (Beveridge, 1941; Kennan et al., 2001; Kennan et al., 2010; Roberts and Egerton, 1969) and *F. necrophorum* has been shown to have a secondary role after the development of disease in some cases (Witcomb et al., 2014). Out of all the bacteria listed above, *D. nodosus* is the only organism that is capable of reproducing the disease when applied as a pure culture (Beveridge, 1941; Egerton and Burrell, 1970).

The prerequisite of the disease is the damage to the interdigital epithelium that may be caused by pebbles, grit, coarse grass, wet weather, water maceration (Green and George, 2008; Gurung, 2006; Moore, 2005; Whittington and Nicholls, 1995) leading to disruption of the skin barrier allowing colonization of secondary bacteria and subsequent damage to the stratum corneum for infection to proceed (Beveridge, 1941).

Footrot is caused by several bacteria and *F. necrophorum* is thought by some to be responsible for predisposition and initiation of the early stages of the disease (Egerton et al., 1969; Roberts and Egerton, 1969). It was postulated that *F. necrophorum* invades

the damaged interdigital epithelium, thus, creating anaerobic microenvironment due to the action of its leukotoxin for the colonization of *D*. *nodosus*. *D*. *nodosus* then invades the interdigital skin via its type IV fimbriae (Egerton et al., 1969; Han et al., 2008; Roberts and Egerton, 1969) and attaches to the surface due to its potential attachment factor Pgr (Calvo-Bado et al., 2011a). The action of *D*. *nodosus* virulent proteases *aprV2* and *aprV5* (Kennan, 2011) and along with the *F*. *necrophorum* synergistically cause progressive and destructive infection of the epidermal matrix and in severe cases causes the hoof horn to separate from the dermis (Egerton et al., 1969). However, if the infection remains localized to the interdigital skin, it leads to the inflammation causing ID, which is the less severe form of the disease (Kaler et al., 2010b). The extent of the damage caused to the foot depends on host genetic factors, environmental factors and virulence of the organism involved (Egerton, 1991). A recent study has reported that *Fusobacterium necrophorum* is a secondary bacterium after the development of the severe footrot and contributes to the severity and duration of the disease (Witcomb, 2012; Witcomb et al., 2014).

The diagnosis of footrot in the field relies on the clinical signs and damage caused to the foot (Egerton et al., 2002). Lameness is the characteristic sign of footrot in sheep. An initial diagnosis is made by examining the feet for the presence of interdigital inflammation and can be confirmed by presence of *D*. *nodosus* rods in a Gram strained smear collected from the foot lesion. Severe cases are associated with separation of the soft tissue from the hoof horn and are often accompanied with a foul smell. Previously, the diagnosis of footrot was based on the isolation of *D*. *nodosus* from foot lesion of the

infected sheep and characterization using molecular methods, but this is often too slow and could lead to spread of disease before the diagnosis is made (Stäuble et al., 2014). The development of virulence tests such as protease thermostability or gelatin gel assay, elastase test, presence of *vap* and *vrl* regions and virulence associated *intA* gene have aided in the diagnosis and are being used in the laboratories. However, a recent study compared these tests on the isolates of *D*. *nodosus* taken from 12 sheep flocks that were suffering from the VFR (Dhungyel et al., 2013). Little agreement between the test results was observed suggesting that these tests are not reliable methods for the correct diagnosis of footrot (Dhungyel et al., 2013). In the UK, the gelatin gel test classified the majority of strains isolated from sheep as virulent, irrespective of the clinical condition of the feet at the time of isolation (Moore, 2005). Therefore, these tests are less informative for the UK situation. Recently, a qPCR assay targeting *D*. *nodosus 16S rRNA* gene was developed and enabled more specific and sensitive detection of *D*. *nodosus* (Frosth et al., 2015). However, to date, the only reliable and more advanced strain differentiation based on the presence of serine proteases *aprV2* and *aprV5* has been developed and is widely used for differentiation of *D*. *nodosus* strains (Dhungyel et al., 2013; Kennan, 2011). Environmental factors have been shown to influence the diagnosis of footrot. In hot and dry weather, the disease is not fully expressed as it requires moist and wet conditions for clinical signs to occur, therefore early detection of footrot is challenging during such conditions (Dhungyel et al., 2013).

A number of diseases cause lameness in sheep and includes contagious ovine interdigital dermatitis (CODD) (Moore et al., 2005b; Wassink et al., 2003). CODD is caused by

spirochetes and is characterized by hemorrhagic lesions around the coronary band, redness and swelling (Collighan et al., 2000; Demirkan et al., 2001; Dhawi et al., 2005). However, treponemes were isolated from sheep with CODD in the UK at two separate occasions (Demirkan et al., 2001; Walker et al., 1995). It is unknown what the role of spirochetes in footrot is, but it has been reported that spirochetes were unable to reproduce the disease when applied in pure cultures (Beveridge, 1936). Further work is warranted to determine their role in footrot progression.

Several other hoof diseases have been reported and include digital dermatitis, infectious pododermatitis, foot abscess, severe virulent ovine footrot and strawberry footrot also known as proliferative dermatitis. It is caused by *Dermatophilus congolensis* and is characterized by lesions around coronet extending to the hock with the formation of scabs. When the crust of the scabs is removed, it forms spots of bleeding that resemble a strawberry (Harriss, 1948). Infectious pododermatis also known as Interdigital necrobacillosis is caused by *F. necrophorum* and affects cattle whilst sheep are rarely affected with the disease (Clark et al., 1985).

Foot abscesses occurs in both sheep and cattle and are caused by invasion of the interdigital epithelium by *Fusobacterium species* accompanied by pyogenic bacteria such as *Trueperella pyogenes* leading to lameness and oozing of pus (Gregory, 1939). The disease is also known as digital suppuration (Gregory, 1939; Thomas, 1962) and infective bulbar necrosis (Roberts et al., 1968).

1.2. Treatment.

The current known effective treatment of footrot is rapid treatment of diseased sheep with systemic antibiotics and topical application of antibiotic spray (Kaler et al., 2010a; Wassink et al., 2010b). This reduces the prevalence, incidence of lameness in sheep (Kaler and Green, 2009a; Wassink et al., 2010b). Intramuscular administration of long acting antibiotics, such as tetracycline and oxytetracycline in combination with topical antibiotic spray of the affected area is highly effective at combating ID and VFR (Kaler et al., 2010a; Kaler et al., 2012). This results in > 95 % recovery and wound healing occurring within 3-4 days (Kaler et al., 2010a; Wassink et al., 2003, 2004).

Foot trimming was previously used for the treatment of footrot but current recommendations suggest that feet should not be trimmed. Routine foot-trimming is associated with increased prevalence and incidence of ID as well as VFR in a flock (Green et al., 2007; Kaler and Green, 2009a; Wassink et al., 2003).

Foot-bathing with antiseptic solutions such as 10 % zinc sulphate and 3% formalin are reported to provide cure rates of 61-77 % in UK sheep flocks (Grogono-Thomas et al., 1994; Parajuli and Goddard, 1989; Winter, 2008). However, foot bathing is reported to increase the prevalence of VFR in ewes (Kaler and Green, 2009a) though it can aid in controlling ID in the lambs (Wassink et al., 2004).

Treatment of footrot in the UK can be achieved by treating sheep with parenteral antibiotics and topical spray within the first three days of becoming lame and lambs with ID can be treated with a topical antibiotic spray alone. If sheep do not respond to the

treatment, they should be treated again with systemic antibiotic and topical spray after two weeks of the initial treatment.

1.2.1. Vaccination.

Vaccination has been a useful tool in protecting populations of sheep against footrot in some countries. The first monovalent whole cell vaccine was developed in 1969. The vaccine showed therapeutic effect and gave protection against homologous challenge (Egerton, 1970). A field trial study evaluated a bivalent vaccine that gave partial protection against infection and vaccinated sheep developed less severe lesions than non-vaccinated flock (Egerton, 1974). The whole cell vaccine incorporating all known serogroups of *D*. *nodosus* failed to control footrot under field conditions. This is because multiple serogroups of *D*. *nodosus* co-existed on a particular farm and the vaccine did not confer adequate protection against mutiple serogroups (Egerton, 1973, 1974). The protective immunoglobulin response of a monovalent whole cell vaccine lasted for approximately 16 weeks (Hunt et al., 1996; Raadsma et al., 1994).

A commercial multivalent vaccine containing eight serogroups was evaluated and showed therapeutic effect and as well gave significant protection against *D*. *nodosus* infection (Hindmarsh, 1989). It was reported that multivalent vaccine gave partial protective against heterologous challenge and the protective immunoglobulin response lasted for only 12 weeks (Raadsma et al., 1994). It has been suggested that the antigenic competition between the serogroups may be responsible for inadequate immunity conferred by multivalent vaccines (Hunt et al., 1996; Raadsma et al., 1994;

Schwartzkoff et al., 1993). The mechanism by which antigenic competition occurs has not been fully studied. However, it has been suggested that antigens compete and inadequately bind to the major histocompatibility complex II binding sites on the antigen presenting cells (Raadsma et al., 1994).

A multivalent recombinant fimbrial vaccine "Footvax" containing serogroups A, B1, B2, C, D, E, F, G, H and I was developed and evaluated in Australia (Hunt et al., 1995; Raadsma et al., 1994; Schwartzkoff et al., 1993). The vaccine conferred partial protection and the protective immunoglobulin titre lasted for less than ten weeks (Schwartzkoff et al., 1993). However, this vaccine is used worldwide as a control measure and is the only multivalent vaccine produced commercially.

Outbreaks of footrot with a mono serogroup of *D*. *nodosus* occurred in some countries such as Bhutan, Australia and India. In Nepal serogroups B and E of *D*. *nodosus* were detected in the sheep flocks (Egerton et al., 2002). To control the disease, two doses of specific fimbrial vaccine were injected in sheep one-month apart and conferred protection for approximately 18 months (Egerton et al., 2002). The use of serogroup specific vaccination was successful in elimination footrot from Nepal, which was confirmed by extensive bacteriological surveillance and anamnestic ELISA (Dhungyel et al., 2001; Egerton et al., 2002). In Bhutan, serogroup B was reported to be associated with the outbreak. A specific monovalent vaccine conferred protection for more than 12 months and no cases of footrot were detected after one year of vaccination and other control methods. Therefore, the use of serogroup specific vaccination in Bhutan contributed to complete eradication of footrot from affected flocks (Gurung, 2006). In

Australia, an outbreak specific vaccination has also been shown to control and eradicate the disease from sheep farms (Dhungyel et al., 2013). In the state of Jammu and Kashmir-India, serogroup B specific whole cell vaccine has also been shown to reduce the prevalence of footrot and confers therapeutic as well as prophylactic response. The protective antibody titre of the vaccine lasted for approximately four months (Unpublished data).

In the UK, multiple serogroups of *D. nodosus* co-exist in individual flocks (Moore et al., 2005a) and the vaccine "Footvax" does not confer protection due to antigenic competition between the serogroups (Dhungyel and Whittington, 2009). The UK farmers report mixed benefits from the vaccine, overall farmers relying solely on vaccine for control are dissatisfied (Wassink et al., 2010a). Therefore, the sole use of "Footvax" is not an ideal control strategy and further research is needed to determine if there are other potential virulence determinants present on the *D. nodosus* chromosome and targeting them in a vaccine could potentially control footrot.

1.2.2. Control and Eradication.

Control of footrot is defined as minimising the impact and occurrence of a disease in a flock whilst not eliminating the causal agent (Green and George, 2008). It is aimed to reduce the prevalence of *D. nodosus* infection in the affected sheep either through vaccination or by using the combination of systemic and topical antibiotics with vaccination. These measures have been successful in achieving long-term control of the disease in the UK. A five-point management plan has been recommended to UK sheep farmers to control lameness on their farms (EBLEX) and includes:

i. Culling of the infected sheep that are repeatedly lame.
ii. Biosecurity measures such as quarantine newly purchased sheep for a period of 28 days.
iii. Rapid injection of all sheep with lameness with long-acting antibiotics and topical antibiotic spray.
iv. Reducing disease challenge by avoiding spread of infection during management practices.
v. Vaccination of the whole-flock using primary and a booster six month apart.

Preventative measures such as frequent foot bathing in zinc sulphate, formalin and copper salts reduce the spread of footrot; vaccination targeting predominant serogroups and treatment with systemic antibiotics are essential control measures (Raadsma and Egerton, 2013).

Eradication is defined as complete elimination of the pathogen from the geographical area (Green and George, 2008) and has been successful in parts of Australia, where the "New South Wales (NSW) footrot strategic plan" was implemented (Egerton et al., 2004). Implementation of the NSW footrot strategic plan significantly reduced the annual losses from AUS $42.6 M to AUS $500,000 (Egerton et al., 2004).

Eradication can be achieved by using a combination of vaccination, treatment, culling of the diseased sheep and strict quarantine to maintain a disease free flock (Wassink et al., 2003, 2004). However, the Australian elimination programme cannot be implemented in the UK because no seasonality (i.e predictable periods of dry weather) is observed and transmission rates are higher during wet periods (Wassink et al., 2003). Also, it is

difficult to completely eliminate the disease from the UK due to the factors such as high annual rainfall, high stocking densities, inability of farmers to transport sheep to the slaughter house for culling; maintenance of strict quarantine (Green and George, 2008; Wassink et al., 2010a; Wassink, 2006). For successful elimination of the disease in the UK sheep farms, it has been recommended that all farmers should strictly adhere to the guidelines such as quarantine, isolation and culling of the infected sheep, maintenance of strong fences to separate diseased sheep from healthy and once the disease prevalence is reduced and/or complete elimination is achieved, a disease free flock should be purchased and reintroduced (Green and George, 2008).

1.3. Microbiology.

1.3.1. *Dichelobacter nodosus*.

D. *nodosus* is a Gram-negative, slightly curved rod shaped aerotolerant anaerobe with characteristic bulged ends. It is 3 – 10 µm long and 0.6 – 0.8 µm wide at the centre and 0.8 µm to 1.2 µm at the ends (Beveridge, 1941). It is cultured on hoof agar plates containing arginine, serine, trypticase, yeast extract, protease peptone (Lab Lemco) and hoof powder 4% and 2% for isolation and subculture respectively (Pitman et al., 1994; Skerman, 1975). The optimum pH for its growth is 7.4 - 7.6. The colonies have a typical "ground glass" appearance (Thorley, 1976) and are classified as B type (beaded), M type (mucoid) and C type (circular). Among the anaerobic bacteria, *D*. *nodosus* has the smallest genome sequenced with the size of 1.4 Mb and contains 1, 389, 350 bp (Myers et al., 2007).

1.3.2. Virulence factors.

A number of characteristics of *D*. *nodosus* have been investigated to determine if they are virulence factors. These include colony characteristics (Depiazzi et al., 1990), presence of pili (Gradin, 1991), and production of thermostable protease (Palmer, 1993). Phenotypic and molecular markers have been used for virulence testing and include the elastase test (D., 1979), the protease thermostability test (Palmer, 1993); the presence of *intA* has been used to screen *D*. *nodosus* isolates because the gelatin gel test sometimes gives false positive results (Cheetham et al., 2006). The above tests do not always discriminate between virulence and benign isolates which suggests that these are not the absolute molecular markers for virulence and it could be that virulence is associated to multiple processes (Calvo-Bado et al., 2011a). Macro-restriction pulsed-field gel electrophoresis (PFGE) has been developed for diagnostic purposes and is reported as a reliable method to discriminate the strains of *D*. *nodosus*. A recently reported AprV2 protease (Kennan et al., 2010) is widely used in the UK and elsewhere to differentiate between virulent and non-virulent isolates of *D*. *nodosus*.

1.3.2.1. Type IV fimbriae.

Fimbraie or pili are non-flagellar, filamentous appendages on the cell surface of bacteria (Figure 2) (van der Woude and Bäumler, 2004). These are strongly antigenic and mediate the K-type agglutination (Stewart, 1973; Walker, 1973). They are constructed from fimbrial/pilin subunit having molecular weight of 16.0 - 19 KDa and contains 140 – 165 amino acid residues (Mattick et al., 1984). The fimbriae are classified as type IV (Billington et al., 1996) and includes the fimbriae of *Neisseria gonorrhoeae*, *D*.

nodosus, *Pseudomonas aeruginosa and Moraxella bovis* (Dalrymple and Mattick, 1987). They share some common characteristics such as polar location on the cell that enables twitching motility and a highly conserved N-terminal domain that contains N-methlyphenylalanine residue.

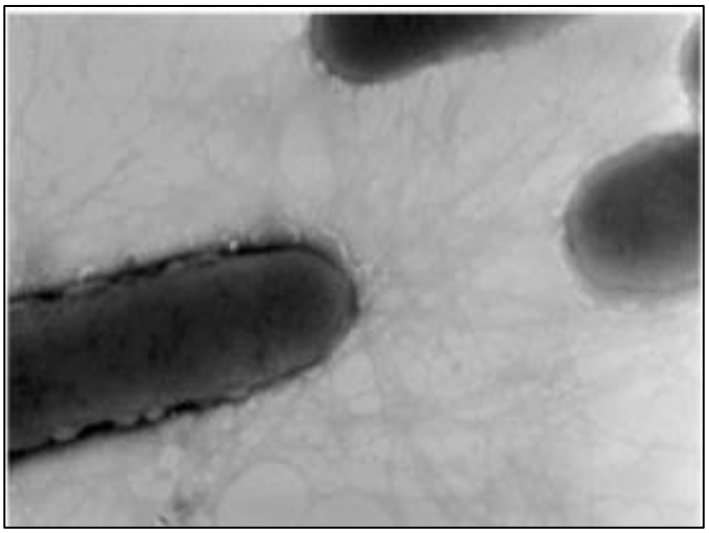


Figure 1.2. Transmission electron microscopy image of surface fimbriae of *Dichelobacter nodosus*. Wild type *D*. *nodosus* (VCS1703A) cells showing the presence of fimbriae. Scale bar: 1 mm. The figure is a modified version of Figure-2 from (Parker et al., 2006).

Antigenic variation among *D*. *nodosus* strains was determined by slide agglutination test. A heat labile surface antigen (K) was responsible for coarse K-type agglutination and a heat stable somatic antigen (O) caused fine agglutination (Egerton, 1973). K-type agglutination was caused due to reaction with the fimbriae (Short et al., 1976).

Essential virulence factors of *D*. *nodosus* are its type IV fimbriae (Han et al., 2008) and its capability to produce extracellular proteases (Kennan et al., 2001; Kennan et al.,

2010). *D. nodosus* fimbriae mediate attachment and adhesion to the epithelial cells and twitching motility (Kennan et al., 2001). Fimbriae are reported to elicit highest immune response and mediates K-agglutination that classified *D. nodosus* into 10 serogroups, A–I, M (Claxton et al., 1983; Ghimire et al., 1998). The serogroups are further subdivided into 18 serotypes, A12, B14, C, D, E12, F12, G12, H12. There is evidence of sequence variation within the FimA protein, which forms the basis of serological diversity in *D. nodosus* (Zhou and Hickford, 2000).

To demonstrate the role of type IV fimbriae in the virulence of *D. nodosus*, a challenge study was performed in Australia using *fimA* mutants. All sheep that were challenged did not suffer from footrot. However, infection with the wild type strain (VCS1703A) having intact fimbraie caused VFR (Kennan et al., 2001). In addition, *fimA* mutants did not produce fimbriae on the cell surface, showed reduced protease activity and did not undergo transformation. The study by Kennan et al. (2001) did not demonstrate whether the inability of *fimA* mutants to cause disease was due to the loss of adherence to the surface, twitching motility or extracelluar protease secretion. However, it was later reported that twitching motility mediated by type IV fimbriae has a key role in the pathogenesis of footrot (Han et al., 2008).

1.3.2.2. Extracellular serine proteases.

Proteases are the major group of enzymes that are essential for the virulence of *D. nodosus*. These enzymes degrade proteins such as keratin, collagen and elastin in the hoof (Kennan et al., 2010). *D. nodosus* secretes three closely related extracellular

subtilin like proteases that are essential virulence factors (Billington et al., 1996; Kortt et al., 1993). These include acidic proteases such as AprV2, AprV5 and BprV, a basic protease secreted by the virulent strains (Kennan, 2011; Lilley et al., 1992). Proteases AprB2, AprB5, BprB are secreted by benign strains and cause ID (synonyms with benign footrot in Australian research) (Kennan et al., 2010). Comparative sequence analysis of the proteases from virulent (*D. nodosus* A198) and benign (*D. nodosus* C305) strains revealed a single amino acid difference (Y92R) between AprV2 and B2 (Riffkin et al., 1995) and between AprV5 and B5 (Riffkin et al., 1993).

The proteases degrade the epidermal layer of the interdigital skin and breaks the skin-horn junction allowing invasion of *D. nodosus* (Kennan et al., 2010). To demonstrate the role of proteases in tissue damage and disease process, mutants of protease genes were constructed from the wild type isolate (VCS1703A) by allelic exchange and characterized for the elastase activity and virulence in sheep pen trails (Kennan et al., 2010). It was observed that *aprV2* mutant did not digest elastin (A test used in Australia for differentiation of virulent and benign strains) and upon complementation with *aprV2,* the ability to digest elastin was restored, thus providing evidence that AprV2, is responsible for the extracellular elastase activity, which is essential for development of the disease. In addition, the results of the pen trials demonstrated that *aprV2*, *aprV5* and *bprV* mutants were unable to cause VFR in sheep but complementation with *aprV2* wild type gene caused footrot, thus fulfilling molecular Koch's postulates (Kennan et al., 2010). AprV2 has also been shown to cause degradation of keratin layer of the ovine hoof (keratin is an integral part of ovine hoof that maintains tissue integrity and confers

physical protection). Hence, AprV2 protease is a major protease which is essential virulence factor of *D*. *nodosus*. It has also been reported that AprV5 is the major extracelluar protease while as BprV has a minor contribution towards the total extracellular activity (Kennan et al., 2010). AprV5 not only causes its own maturation but also responsible for cleaving AprV2 and BprV from their precursor molecule to their mature form that are active (Han et al., 2012). In their mature form, *D*. *nodosus* secretes the proteases into the external environmental. It has been hypothesized that these proteases cause digestion of host (sheep) protein into amino acids, thus providing an external source of amino acids to *D*. *nodosus*. However, to date, the exact role of these proteases in the virulence of *D*. *nodosus* unclear and should be studied further.

Some molecular markers of virulence such as *vap* (virulence associated proteins) and *vrl* (virulence related locus) (Katz et al., 1991; Rood et al., 1996) and *intA* gene (Cheetham et al., 2006) have also been discovered, but their precise role in the virulence is remained to be delineated. Recent work discussed above has provided definitive evidence using knock out strain that the twitching motility (Han et al., 2008) and proteases *aprV2, aprV5, bprV* (Han et al., 2007) are essential virulence determinants.

1.3.2.3. Other potential virulence determinants - Pgr Locus.

The Pgr locus corresponds to a large repetitive secreted protein identified as a putative virulence factor DNO_690 (Calvo-Bado et al., 2011a; Myers et al., 2007). The genome sequence identified a virulence factor with regions of atypical nucleotide composition. A number of putative secreted toxin like proteins were identified (RTX like) which could

cause necrosis in the foot (Myers et al., 2007). The DNO_690 also had atypical nucleotide composition and 32 nine amino acid repeat units indicated a putative role in adhesion to the extracellular matrix of the foot and apparently only occurred in virulent strains (Myers et al., 2007). Calvo-Bado et al.(2011) further developed this hypothesis and named this repetitive secreted protein Pgr for proline glycine repeat (Calvo-Bado et al., 2011a). Evidence suggests that Pgr is a polymorphic protein having a molecular weight of 100 to 120 KDa. Analysis of the sequence revealed that *pgr* gene consists of two variants, *pgrA* and *pgrB*, which were diverse within *D*. *nodosus* strain collections. The genes *pgrA* and *pgrB* have repeat regions, which are hypervariable. In *pgrA*, there are two tandem repeat regions, R1 (glycine rich) and R2 (proline rich) (Figure 1.3). The number of these tandem repeats in the R1 and R2 regions vary in different strains of *D*. *nodosus*. In *pgrA*, the number of R1 repeats varies between 1 – 29 as observed for strains VCS1006 having one tandem repeat and VCS1137 having 29 repeats (Calvo-Bado et al., 2011a). Similarly, the R2 repeats vary between 1 – 16 with BS1 having one tandem repeat and VCS1703A having 16 repeats. Analysis of *pgrB* showed that it contained R3, R4 and R5 repeat regions, which are hypervariable (Figure 1.3). The hypervariability in the tandem repeat copy number provides evidence of Pgr as a polymorphic protein.

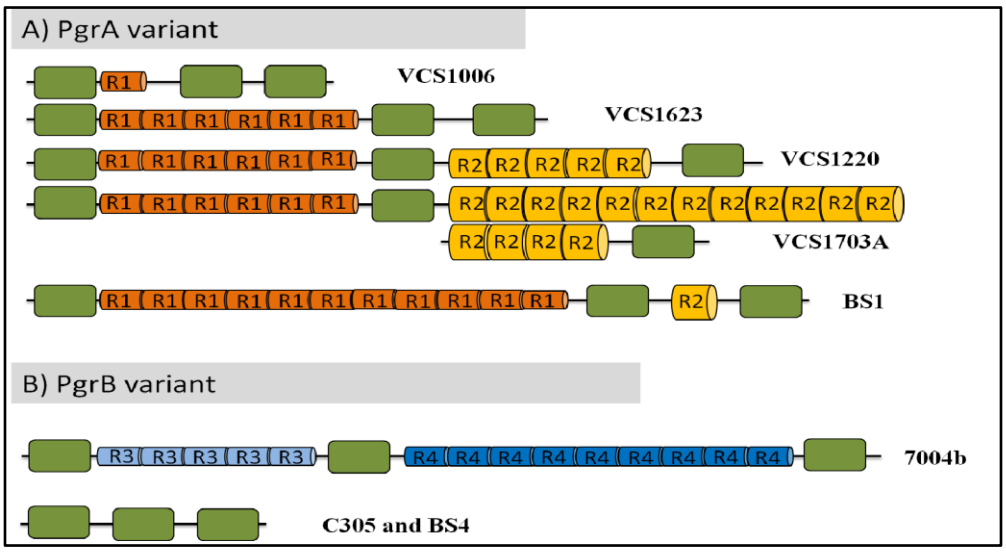


Figure 1.3. Diagrammatic representation of *pgrA* and *pgrB* in *D. nodosus* illustrating the region of tandem repeats. (A) *D. nodosus* strains VCS1006, VCS1623, VCS1220, VCS1703A and BS1 have *pgrA* with variable number of tandem repeats. (B) *D. nodosus* strains C305 and BS4 have *pgrB* and tandem repeats vary in each strain. The figure is a modified version of Figure-1 from Calvo-Bado *et al*. (2011a).

Calvo-Bado et al. (2011) postulated that the polymorphisms in Pgr protein might result from the host's selective immune pressure due to the protein being partially exposed at the surface of the cell (Calvo-Bado et al., 2011a). Though it is not clear how tandem repeats are generated and maintained in bacteria but they may arise due to mutation, transformation and recombination (Bzymek and Lovett, 2001; Verstrepen et al., 2005). It has been reported that during clinical or subclinical infections, multiple strains of *D. nodosus* co-exist in both the individual feet and flock (Claxton et al., 1983; Moore et al., 2005a).

During transformation and homologous recombination, antigenic variations in *D. nodosus* are likely to occur (Kennan et al., 2003). Therefore the opportunities for

extensive genetic recombination are mainly due to the occurrence of recombinant plasmids (5.1 kb plasmid pDN1 reported by Whittle *et al*., 2000), the competence of cells and their natural transformation (Kennan et al., 2003; Whittle et al., 2000) combined with the mixed populations found in situ. There is evidence of extensive variation in chromosomal genes as recently reported for *fimA*, which resulted in altered serotypes but this was attributed to the accumulation of point mutations (Bhat et al., 2012). A plausible hypothesis for *pgrA* polymorphism is that gene transfer and recombination within the *pgr* regions could be responsible for the variants observed (Calvo-Bado et al., 2011a).

Bioinformatic analysis of *pgrA* indicated that it contained two putative transmembrane localization domains; an alpha chain which is made up of collagen and collagen-like anchor motif present on the surface of the cell (Calvo-Bado et al., 2011a). This generates a protein that is collagen like and has an anchor motif on the cell surface, which has previously been shown to aid in the attachment of pathogenic bacteria to the host. A recent study using linear and circular dichorism suggested *pgrA* to have an alpha helical structure and its interaction with collagen (Stoltz, 2013). These results support the hypothesis that *pgrA* may be responsible for attachment of *D*. *nodosus* to the foot but further work is needed to prove this.

Calvo-Bado *et al* (2011) tested 100 isolates from the sheep affected with VFR in the UK, Australia, Sweden and benign footrot in Western Australia. Both variants of *pgr* were detected in the isolates from UK, Australia and Sweden. However, in Western

Australian collection, *pgrB* variant was only detected suggesting *pgrA* variant may be associated with VFR and *pgrB* with ID.

1.4. The *D. nodosus* genome.

The genome of *D*. *nodosus* was sequenced and reported in 2007 (Myers et al., 2007) and it has the smallest genome of approximately 1.4 MB. Considerable evidence for lateral gene transfer is evident and the regions affected are associated with virulence in most cases. This may relate to the capacity of *D*. *nodosus* for transformation. As discussed previously, the *pgrA* gene is one of many putative virulence determinants identified. In the study a number of putative virulence factors were identified by transcriptional profiling and bioinformatic analysis and 80 immunogenic proteins were identified by screening against ovine antisera and were recommended as candidate antigens for cross protective vaccine. Further genome analysis has been achieved by sequencing a number of diverse strains (n=103) from Australia, Bhutan, Nepal, India, Denmark, Norway, Sweden and UK (Kennan, 2014). All *D*. *nodosus* isolates were highly conserved with > 95 % sequence identity. Comparative genome analysis of 103 sequences revealed *D*. *nodosus* strains divided into two distinct clades I and II based on the analysis of single nucleotide polymorphisms (SNP's) of 31,627 nucleotides (Kennan, 2014). A correlation between clade I with virulent phenotype and clade II with benign phenotype based AprV2 and AprB2 was also observed. The division into clade I and clade II did not take the geographical origin of the isolates into account. However, the isolates from a particular geographical area present in either of the clades clustered separately.

1.5. Transmission of *D. nodosus*

D. *nodosus* can be transmitted directly between sheep through the contaminated pasture, bedding, faeces and soil (Muzafar et al., 2015). The transmission of footrot depends upon a number of factors such as, climatic conditions, temperature (generally >10 ^{o}C) and nature of the particular isolate (Whittington, 1995; Whittington and Nicholls, 1995). Warm and wet weather conditions are favourable for the occurrence of footrot (Smith *et al*., 2014), leading to milder form of footrot in spring (Wassink et al., 2003). Reports show that *D*. *nodosus* does not persist for long enough in the environment but have been observed to survive for two weeks during optimum conditions (warm and damp) (Beveridge, 1941; Whittington, 1995). The disease is determined to be seasonal in Australia, where there are more predicted periods of transmission (Green and George, 2008), whereas not much is observed with respect to its relation with season in UK (Wassink et al., 2003). *D*. *nodosus* exists in the feet of sheep and is further transmitted from one sheep to other via pasture (Moore et al., 2005a).The potential risk factors associated with occurrence of footrot in UK include high rainfall, winter housing, high stocking densities, lowland farms and coarse grass (Moore et al., 2005a; Wassink et al., 2003, 2004).

This transmission between feet and sheep is thought to occur indirectly via contaminated pasture or housed floors (Beveridge, 1941; Whittington, 1995). In absence of any source for *D*. *nodosus* in the environment, the infected sheep and goats are thought to act as a sole reservoir (Whittington, 1995) and it has been shown that asymptomatic infection may also occur (Depiazzi et al., 1998). Recent advances enable detection of strain

differences using molecular markers of DNA rather than cultured samples. These include *pgr* typing (Calvo-Bado et al., 2011a) and multi locus variable number tandem repeat analysis (MLVA) (Russell et al., 2014). MLVA is a typing approach that discriminates individual isolates based on the enumeration of four polymorphic tandem repeats within the genome. Comparing MLVA profiles can provide information on population diversity and genetic relatedness (Russell et al., 2014) aiding in epidemiological studies.

A recent study characterised the *in vivo* strain diversity of *D*. *nodosus* between ewes and lambs using *pgrA* and MLVA typing. The study investigated how soon after birth *D*. *nodosus* could be detected on the feet of newborn lambs, and the potential role-played by the environment in pathogen dissemination. The results indicated that environment acts as a reservoir for infection of newborn lambs exposed to bedding contaminated with *D*. *nodosus* caused by ewes sharing the same enclosure (Muzafar et al., 2015).

1.6. Survival of *D. nodosus*.

The first study on the survival of *D*. *nodosus* outside of the host was reported in 19th century by Brown (1892) in England who demonstrated that *D*. *nodosus* survived on wet pasture and caused footrot in sheep two days after the removal of infected sheep from the pasture (Brown, 1892). In the 20th century, Marsh and Tunicliff (1934) reported that the *D*. *nodosus* survived for 15 days in dry pens and 30 days when the pens were continually wetted (Marsh and Tunnicliff, 1934). Survival away from the host is dependent on moist, mild conditions (Graham and Egerton, 1968) and *D*. *nodosus* is

reported to survive for no more than two weeks on pasture during optimum conditions of warm and damp (Beveridge, 1941; Whittington, 1995). It has been postulated that *D. nodosus* can stay dormant in the hoof when the conditions are dry and upon return of favorable milieu (warm and damp), the disease progression occurs (Beveridge, 1941; Mohler and Washburn, 1905).

A recent a study using qPCR showed that *D. nodosus* can survive up to 14 days in soil in a laboratory at 5 ^{o}C and for a further 24 days if the soil was amended with hoof powder. A significant decrease in survival time was observed in soil with the increase in temperature of microcosms up to 15 ^{o}C, however moisture content was not stated (Cederlof et al., 2013). Also, it is not known if this survival was at a dose that could cause disease in sheep.

Various organisms such as *Campylobacter jejuni*, *Campylobacter. coli* and *Vibrio species* are capable of surviving in the environment in viable but non-culturable state (Rollins and Colwell, 1986). However, it is unknown whether *D. nodosus* can survive in the environment in viable form but not in a culturable form.

1.7. Hypothesis, Aims and Objectives.

The overarching aim of the research was to study transmission routes for *D*. *nodosus* and establish the role-played by the environment in dissemination of the disease and consider strain diversity within an establish flock.

1.7.1. Hypothesis-A.

Transmission occurs via contaminated ground between ewes and their lambs in the first case and secondly between lambs in the field and similarly between ewes.

Aim-A1: To study lateral transmission of *D*. *nodosus* between ewes and their lambs.

Objective: Use molecular typing tools to prove transmission of *D*. *nodosus* between ewes and lambs (10 of each) immediately after birth.

Cloning *pgrA* from the community DNA samples taken from 10 ewes and 10 lambs and comparing MLVA profiles by length heterogeneity.

Aim-A2: To study survival of *D*. *nodosus* under controlled environmental conditions.

Objective: Investigate the extent of *D*. *nodosus* survival in environment and factors influencing viability of cells; determine impact of soil type and moisture content.

Use soil microcosms with different types of soils and varying moisture content, isolation of *D*. *nodosus* by culture, detection of 16S rRNA gene PCR and enumeration of *D*. *nodosus* load by a qPCR assay targeting *rpoD* gene.

1.7.2. Hypothesis B.

The population of *D*. *nodosus* in the UK colonizing sheep can be divided into two groups virulent and benign which correlates with the distribution of *pgrA* and *pgrB*.

Aim B: To attempt a genome wide study of selected isolates and compare these with global population diversity and investigate the distribution of virulence determinants including *pgr*.

Objective B1: Study diversity of *pgr* and examine the association of specific *pgr* variants with other markers such as *aprV2* and *aprB2* by SNP analysis of whole genomes.

Objective B2: Analyse expression of *pgrA* and *pgrB* in *D*. *nodosus* in vitro.in response to hoof horn.

CHAPTER 2

The role of the environment in transmission of *Dichelobacter nodosus* between ewes and their lambs

2.1. Abstract

Dichelobacter nodosus (*D*. *nodosus*) is the essential causative agent of footrot in sheep. The current study investigated when *D*. *nodosus* was detectable on the feet of newborn lambs and possible routes of transmission. A specific qPCR was used to detect and quantify the load of *D*. *nodosus* in foot swabs of lambs at birth and 5 -13 hours post-partum, and their mothers 5-13 hours post-partum; and in samples of bedding, pasture, soil and faeces. *D*. *nodosus* was not detected on the feet of newborn lambs swabbed at birth, but was detected 5-13 h after birth, once they had stood on bedding containing naturally occurring *D*. *nodosus*. Multiple genotypes identified by cloning and sequencing a marker gene, *pgrA*, and by multi locus variable number tandem repeat analysis (MLVA) of community DNA from swabs on individual feet indicated a mixed population of *D*. *nodosus* was present on the feet of both ewes and lambs. There was high variation in *pgrA* tandem repeat number (between 3 and 21 repeats), and multiple MLVA types. The overall similarity index between the populations on ewes and lambs was 0.45, indicating moderate overlap. Mother offspring pairs shared some alleles but not all, suggesting lambs were infected from sources(s) other than just their mother's feet. We hypothesise that *D*. *nodosus* is transferred to the feet of lambs via bedding containing naturally occurring populations of *D*. *nodosus*, probably as a result of transfer from the feet of the group of housed ewes. The results support the hypothesis that the environment plays a key role in the transmission of *D*. *nodosus* between ewes and lambs.

2.2. Introduction.

Footrot is an economically important disease of sheep. The aerotolerant anaerobe *Dichelobacter nodosus* (*D*. *nodosus*) is the essential causative agent (Beveridge, 1941) and *Fusobacterium necrophorum* has been suggested as a secondary bacterium after the development of disease (Beveridge, 1941; Witcomb et al., 2014). The disease is present worldwide and accounts for annual losses of between £24 and £84 million to the UK sheep industry alone (Nieuwhof and Bishop, 2005; Wassink et al., 2010b). The severity of ovine footrot can vary from mild interdigital dermatitis (synonymous with benign footrot in Australian research) to virulent footrot causing severe under-running of the hoof horn with separation from the underlying tissue (Stewart, 1989). *D*. *nodosus* can be detected on the feet of sheep with no sign of disease (Calvo-Bado et al., 2011b; Witcomb et al., 2014), but the load is higher both before and during episodes of interdigital dermatitis and virulent footrot than on healthy feet (Witcomb et al., 2014).

Temporal clustering of footrot between mothers and offspring was observed in a state transition study of factors associated with development of, and recovery from, footrot. Given that families cluster spatially this suggests spatiotemporal transmission of *D*. *nodosus* between family members (Kaler et al., 2010b). *D*. *nodosus* has been isolated from pasture and barns where sheep are kept, indicating that contamination of the environment occurs (Witcomb, 2012). Contaminated holding areas have also been shown to cause disease in sheep put into such environments up to two weeks from initial seeding (Beveridge, 1941). Recent work has indicated that *D*. *nodosus* can survive up to 14 days at 5 ^{o}C in soil, and at least 24 days when hoof material was present (Cederlof et

al., 2013) and under certain conditions, *D*. *nodosus* has survived for at least 40 days in soil microcosms (Muzafar et al., 2015b), however, further work is required to determine if survival is at a dose that could cause disease in sheep.

Multiple strains of *D*. *nodosus* detected by serogroup typing have been reported to co-exist in individual feet during subclinical and clinical infections (Claxton et al., 1983; Hindmarsh and Fraser, 1985; Jelinek et al., 2000; Moore, 2005). Molecular detection of strain differences is now possible using typing of the *pgr* locus and by MLVA of *D*. *nodosus* (Calvo-Bado et al., 2011a; Russell et al., 2014).

The aims of this study were to investigate whether *D*. *nodosus* was present on the feet of newborn lambs at or after birth and the potential role played by the environment in pathogen transmission.

2.3. Materials and methods.

2.3.1. Selection of animals.

In April 2011 ten ewes with no clinical signs of disease and one lamb per ewe were convenience selected from a flock of 99 Mule and Suffolk crossbred ewes. Ewes were housed on the 28th March 2011, and samples collected on the 1st – 6th April 2011 (Table 2.1). Lambs were born in a large communal straw bedded pen, ewes and their lambs were moved to individual pens once the ewe had given birth to all her lambs. Sampled lambs were marked with tape so they could be identified for subsequent sampling.

Table 2.1. Ewes and lambs included in the study, with regards to, date of birth (D.O.B.) and time of sampling after birth.

Ewe ID	Lamb ID	D.O.B	Time of sampling after birth (hours)
1	1	6-Apr-11	7
2	2	5-Apr-11	8
3	3	4-April-11	11
4	4	4-April-11	11
5	5	1-April-11	5
6	6	5-Apr-11	8
7	7	5-Apr-11	13
8	8	5-Apr-11	7
9	9	5-Apr-11	7
10	10	5-Apr-11	10

(D.O.B: Date of birth)

2.3.2. Collection of environmental and foot swab samples.

Environmental samples were taken in March prior to lambing and included swab samples of thirty fresh hoof prints in soil, four soil samples from the area around water containers, ten samples of faecal material on the ground and compacted in the interdigital space and three straw samples collected from the storage area. In April, ten straw bedding samples were collected from the communal pen where pregnant ewes were housed. All samples were stored at 4 °C for transportation and at -80 °C until analysed. All four feet of each lamb was swabbed using sterile cotton swabs (EUROTUBO collection swab; Delta lab, Rubi, Spain) directly after birth and before the

lamb touched the ground. The lamb and its dam were sampled 5 - 13 h later once the lamb had stood and been transferred, with its mother, to an individual pen. Swabs were stored at 4 °C for transportation and at -80 °C on arrival at the laboratory.

2.3.3. Detection limit assay by direct PCR and nested PCR from swabs.

The *D. nodosus* strain VCS1703A was used as a positive control for all PCR reactions. To determine the PCR detection limits, cells were harvested from a 5 d culture grown on 2 % hoof agar, and tenfold serial dilutions (10^{-1} to 10^{-10}) were made in triplicate in sterile phosphate buffered saline (PBS). The numbers of cells in the initial concentration and all dilutions were counted using a haemocytometer. Sterile swabs were inoculated with 500 µl of each dilution, and frozen at -20 °C to produce swabs containing a known bacterial load. Microbial DNA was extracted from swabs as described below and the DNA used to determine assay detection limits.

2.3.4. DNA extraction from swabs.

Total genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, GmbH and Co, Düren, Germany) with modifications. Swabs were thawed at 4 °C and 400 µl of buffer T1 was added followed by 40 µl of proteinase K. The samples were vortexed twice for 5 sec and incubated for 10 min at 56 °C. The mixtures were transferred to microcentrifuge tubes and 400 µl of buffer B3 was added. The samples were vortexed twice for 5 sec and incubated for 5 min at 70 °C then allowed to cool before adding 400 µl of 100% ethanol. The samples were again vortexed twice and the supernatant transferred to a NucleoSpin Tissue column and centrifuged at 11,000 × *g* for

1 min. The flow-through was discarded, the membrane was washed with 500 µl of buffer B5 and centrifuged at 11,000 × *g* for 1 min. The flow-through was again discarded, the column was washed with 600 µl of buffer B5 and centrifuged at 11,000 × *g* for 1 min. The flow-through was again discarded and the membrane dried by centrifugation at 11,000 × *g* for 1 min to remove residual ethanol. The DNA was eluted into 40 µl of elution buffer, warmed to 70 °C and centrifuged at 11,000 × *g* for 1 min and the resultant DNA was stored at -20 °C.

2.3.5. DNA extraction from soil and faeces.

DNA was extracted from soil and faecal samples (one gram each) using the Fast DNA Spin Kit for soil (QBiogene, Carlsbad, CA, USA) according to the manufacturer's instructions, and eluted in 70 µl DES (DNase/Pyrogen Free Water). Sterile soil (autoclaved twice at 121 °C for 15 min) was used as negative controls for each set of extractions. The resultant DNA was stored at -20 °C.

2.3.6. DNA extraction from bedding.

One gram of each of ten bedding samples was thawed, and suspended in 40 ml of transport buffer (sterile PBS containing 20 mM Na_2 EDTA; pH 8.0). The samples were shaken for 1 hour at 37 °C followed by centrifugation for 15 min at 13,523 x *g* at 4 °C. The supernatant was removed and the pellet resuspended in 2 ml sterile PBS. DNA was extracted from 200 µl of this solution using the NucleoSpin Blood kit (Macherey-Nagel, GmbH and Co, Düren, Germany) according to the manufacturer's recommendations, and DNA was stored at -20 °C.

2.3.7. End point and nested PCR.

PCR amplifications were performed using an Eppendorf vapo.protect Mastercycler (Eppendorf, Hamburg, Germany). Each 50 µl reaction contained 25 µl Promega PCR master mix (Promega, Southampton, UK), 1.0 µl each of primers Cc and Ac [10 mM] (La Fontaine et al., 1993) (Table 2.2), 2.5 µl of dimethyl sulfoxide (Fisher Scientific, Leicestershire, UK), 2 µl bovine serum albumin [10 mg ml^{-1}] (Sigma-Aldrich Ltd, Poole, Dorset, UK), 16.5 µl of nuclease free water and 2 µl of template DNA. For direct detection of *D. nodosus*, PCR was performed using Cc and Ac primers (La Fontaine et al., 1993) (Table 2.2) under the following conditions: 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 1 min, 60 °C for 45 sec and 72 °C for 2 min and a final extension step of 72 °C for 5min. Samples that were negative using this approach, were tested further using nested PCR. In the first round *16S rRNA* universal primers 27F and 1525R (Table 2.2) (Baker et al., 2003; Lane, 1991) were used in the conditions described above but with an annealing temperature of 55 °C, 1 µl of this product was used in the second round of PCR as described above. The PCR products were visualised under UV light.

Table 2.2. All primers and probes used in the study.

Primer (5'-3')	Sequence	Expected size in VCS1703A (BP)	Reference
Cc	TCGGTACCGAGTATTTCTACCCAACACCT	783	(La Fontaine et al., 1993)
Ac	CGGGGTTATGTAGCTTGC	783	(La Fontaine et al., 1993)
27F	AGAGTTTGATCMTGGCTCAG	1500	(Lane, 1991); (Baker et al., 2003)
1525R	AAGGAGGTGWTCCARCC	1500	(Lane, 1991); (Baker et al., 2003)
pgrAF1	CCTGCACCATGCTTGTTAAA	290	(Calvo-Bado et al., 2011a)
pgrAR1	GCTGTTGGTGGTTTGGCTAT	290	(Calvo-Bado et al., 2011a)
M13F	GTAAAACGACGGCCAG	N/A	Supplied in the cloning kit
M13R	CAGGAAACAGCTATGAC	N/A	Supplied in the cloning kit
DNTR02F	(6FAM)-GATCCATCGTTTCATCGTCA	549	(Russell et al., 2014)
DNTR02R	CGCACTTTAGCCGTTATGTTT	549	(Russell et al., 2014)
DNTR09F	(VIC)-GGCGTAAACGAAATGCCTAA	987	(Russell et al., 2014)
DNTR09R	ATCGGCGGAAGATTGTCTC	987	(Russell et al., 2014)
DNTR10F	(NED)-CCGTCTATCCACCCGATTTA	626	(Russell et al., 2014)
DNTR10R	TTGAACCGCGTCACTATCAG	626	(Russell et al., 2014)
DNTR19F	(PET)-CCCGTCGAATCACTCCAG	854	(Russell et al., 2014)
DNTR19R	GGTAGCGCCGAAGAAAGA	854	(Russell et al., 2014)
rpoDF	GCTCCCATTTCGCGCATAT	61	(Calvo-Bado et al., 2011b)
rpoDR	CTGATGCAGAAGTCGGTAGAACA	61	(Calvo-Bado et al., 2011b)
rpoD Taqman probe	(6FAM)-CATTCTTACCGGKCG-(BBQ)	61	(Calvo-Bado et al., 2011b)
pgrAF	CATGAATGATAATATTTACCTTTTCGTT	298	(Muzafar et al., 2015a)
pgrAR	AAGATTGATGATGCTCCAGAAGAAG	298	(Muzafar et al., 2015a)
pgrA Taqman probe	(6FAM)-CCTGCACCATGCTTGTTAAACTCT AATTTT-(BBQ)	298	(Muzafar et al., 2015a)
pgrBF	AAAGGTGATCTCAACTGTATCGTCAT	N/A	(Muzafar et al., 2015a)
pgrBR	AATYARCARMGCCARAATTAGAGCTTAAT	N/A	(Muzafar et al., 2015a)
pgrB Taqman probe	(6FAM)-TTTACCCGCACCGTKCT-(BBQ)	N/A	(Muzafar et al., 2015a)

FAM- *Carboxyfluorescein*, BBQ (Black Berry Quencher). BP is the size of fragment in base pairs

2.3.8. Quantitative PCR of *Dichelobacter nodosus*.

The load of *D*. *nodosus* was determined using the Applied Biosystems 7500 Fast real-time detection system (Applied Biosystems, Warrington, UK). The qPCR targeted the *rpoD* gene (RNA polymerase sigma 70 factor, single copy number in *D*. *nodosus* genome) as described previously (Calvo-Bado et al., 2011b). All PCR reactions were performed in triplicate and each contained 12.5 µl TaqMan Universal Master Mix (Applied Biosystems, Warrington, UK), 2.25 µl each of *rpoDF* and *rpoDR* [10 pmol μl^{-1}], 0.625ml *rpoD* probe [10 pmol μl^{-1}] (Table 2.2), 1.25 µl bovine serum albumin [10 mg ml^{-1}], 5.375 µl nuclease free water and 1 µl of template DNA. DNA dilutions of 1:10 were also used to investigate potential inhibitors of the reaction. In addition, known concentrations of target DNA were added to negative samples as internal controls. A non-template control (nuclease free water) was included in triplicate in all PCR reactions. The reaction was carried out under the following conditions: one cycle at 50 ^{o}C for 2 minutes, one cycle at 95 ^{o}C for 10 minutes, followed by 40 cycles at 95 ^{o}C for 15 sec and 55 ^{o}C for 1 min. The *rpoD* copy number was estimated based on the standard curve obtained from analysis of ten-fold serial dilutions of DNA extracted from *D*. *nodosus* strain VCS1703A.

2.3.9. Quantitative PCR assay for *pgrA* and *pgrB*.

A fluorescent PCR was designed to increase the sensitivity of detection of *pgrA* and *B* (Table 2.2). The specificity of the *pgrA* and *B* primers was tested against genomic DNA isolated from 11 bacterial species and from a range of diverse environmental samples. *D*. *nodosus* DNA from VCS1703A was used as a positive control and genomic DNA

from *E. coli* and sterile water as negative and no target controls respectively (Table 2.3, Appendix 1). The cycling conditions were modified slightly from those described above by reducing the number of cycles to 38 and 36 for *pgrA* and *pgrB* respectively.

2.3.10. Cloning and sequencing of *pgrA* amplicons.

The *pgrA* gene was amplified using the primers *pgrA*F1 and *pgrA*R1 (Table 2.2; (Calvo-Bado et al., 2011a) from the DNA extracted from the foot swabs of five ewes (n = 14 feet) and their lambs (n = 10 feet) and cloned using the TOPO TA Cloning Kit (Invitrogen Ltd., Paisley, UK). The cloning reactions were set up following the manufacturer's recommendations. Transformations were carried out using chemically competent (TOP10) *E. coli* cells (Invitrogen Ltd., Paisley, UK) with 50 μg ml^{-1} kanamycin. One-hundred microliters of the resulting solution was cultured on LB plates containing 50 μg ml^{-1} kanamycin. Fifty colonies per sample were inoculated into individual wells of a 96-well plate, each containing 50 μl sterile water. The samples were heated to 75 °C for 10 min, and 1 μl of this solution was used as a template for PCR, resulting in analysis of 1,200 transformants. The PCR products were run on a 1% high resolution agarose gel and visualised under UV light. The clones that showed variation in size within each foot were inoculated into LB media to provide sufficient biomass for plasmid DNA extraction using the Qiagen MiniPrep kit by following the manufacturer's recommendations (Qiagen, West Sussex, and UK). The plasmid DNA was digested using EcoR1 and sequenced by GATC Biotech (London, UK) using the supplied M13f/M13r primers (Invitrogen, Paisley, UK; Table 2.2). All sequences have been deposited in GenBank (Appendix 2).

Table 2.3. ***pgrA*****,** ***pgrB*** **and DNTR primer specificity test using DNA from the environmental samples and genomic DNA from different bacterial species.**

DNA from the bacterial isolates and environmental samples	*pgrA*	*pgrB*	DNTR02	DNTR09	DNTR10	DNTR19
Dichelobacter nodosus **(VCS1703A)**	+	-	+	+	+	+
Dichelobacter nodosus **(C305)**	-	+	N/A	N/A	N/A	N/A
BCG Tuberculosis	-	-	-	-	-	-
DNA isolated from a slaughter house dust from Tanzania	-	-	-	-	-	-
DNA isolated from fresh bedding	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-
Fusobacterium necrophorum	-	-	-	-	-	-
Lactococcus garvieae	-	-	+	-	-	-
Leishmania donowani	-	-	-	-	-	-
Mycobacterium intracellulare	-	-	-	-	-	-
Prevotella oralis	-	-	-	-	-	-
Propionibacterium acnes	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-
Staphylococcus intermedius	-	-	+	-	-	-
Staphylococcus pseudintermedius	-	-	+	-	-	-
Synechococcus **species**	-	-	+	-	-	-
Sterile Water (Negative control)	-	-	-	-	-	-

(*pgrA* and *pgrB* primers were tested by qPCR and DNTR primers were tested by an end point PCR). (+): weakly positive.

2.3.11. Multi Locus VNTR analysis.

The four *D*. *nodosus* tandem repeat (DNTR) loci were amplified individually as described previously (Russell et al., 2014) (Table 2.2) from the DNA isolated from the feet of all ewes and lambs. Primer specificity was tested against genomic DNA isolated from 11 bacterial species and from a range of diverse environmental samples (Table 2.3). *D*. *nodosus* strain VCS1703A was included as a positive control for all PCR reactions. The amplified products from the community DNA were pooled in the ratio of 1:1:1:1 and submitted for fragment analysis to the University of Dundee (DNA Sequencing & Services, Dundee, Scotland). GeneScan 1200 LIZ dye (Applied Biosystems, Warrington, UK) was used as a size standard in fragment analysis. The data obtained were processed using Peak Scanner Software (Applied Biosystems, Warrington, UK) and analysed using T-REX (Culman et al., 2009) with a minimum fragment length cut off value of 500 bp, peak height baseline threshold of 40 and bin range of 4 bp.

2.3.12. Statistical analysis.

To determine the strain overlap between all ewes and lambs defined by MLVA, the coincidence index of overlap was calculated using the formula: $C = 2B / (E + L)$, where C = Coincidence index of overlap, B = occurrence of the same allele in ewes and lambs, E = occurrence of the allele in ewes, L = occurrence of the allele in lambs. Results can range from C = 0, no overlap between ewes and lambs to C = 1, identical strains occur in both ewes and lambs (Dice, 1945).

The *rpoD* copy number in ewes and lambs was not normally distributed. Therefore, a Mann Whitney U test (Mann and Whitney, 1947) was used to test for differences in copy number between ewes and lambs.

2.4. Results.

2.4.1. *D. nodosus* copy number detection limit and its persistence in the environment.

End point PCR and qPCR data of the inoculated swabs suggested that the minimum detection level was 10^4 cells and 10^2 *rpoD* genome equivalents (i.e. 10^2 cells) per swab respectively assuming 100% DNA recovery (Table 2.4). Below this concentration, detection was not reproducible.

Table 2.4. Detection limit of inoculated swabs by endpoint and qPCR.

Concentration of cells added (Enumerated by haemocytometer)	Detection by endpoint PCR	Detection by qPCR	Detection by nested PCR
$2.34x10^9$	+	+	+
$2.34x10^8$	+	+	+
$2.34x10^7$	+	+	+
$2.34x10^6$	+	+	+
$2.34x10^5$	+	+	+
$2.34x10^4$	+	+	+
$2.34x10^3$	-	+	+
$2.34x10^2$	-	+	-
$2.34x10^1$	-	-	-
$2.34x10^0$	-	-	-

D. nodosus was detected by end point PCR in 5 / 10 faecal samples / balls from the interdigital space, 2 / 10 straw bedding samples collected after ewes were housed, 5 / 30

fresh hoof prints and 2 / 4 soil samples taken from the areas surrounding water containers. However qPCR analysis revealed that *D*. *nodosus* was present at loads of 10^2 to 10^4 *rpoD* genome equivalents per gram in all the used straw bedding samples and 10^3 to 10^4 *rpoD* genome equivalents per gram in all the faecal samples. Quantitative PCR also confirmed that *D*. *nodosus* was not detectable in the three stored straw samples (Figure 2.1).

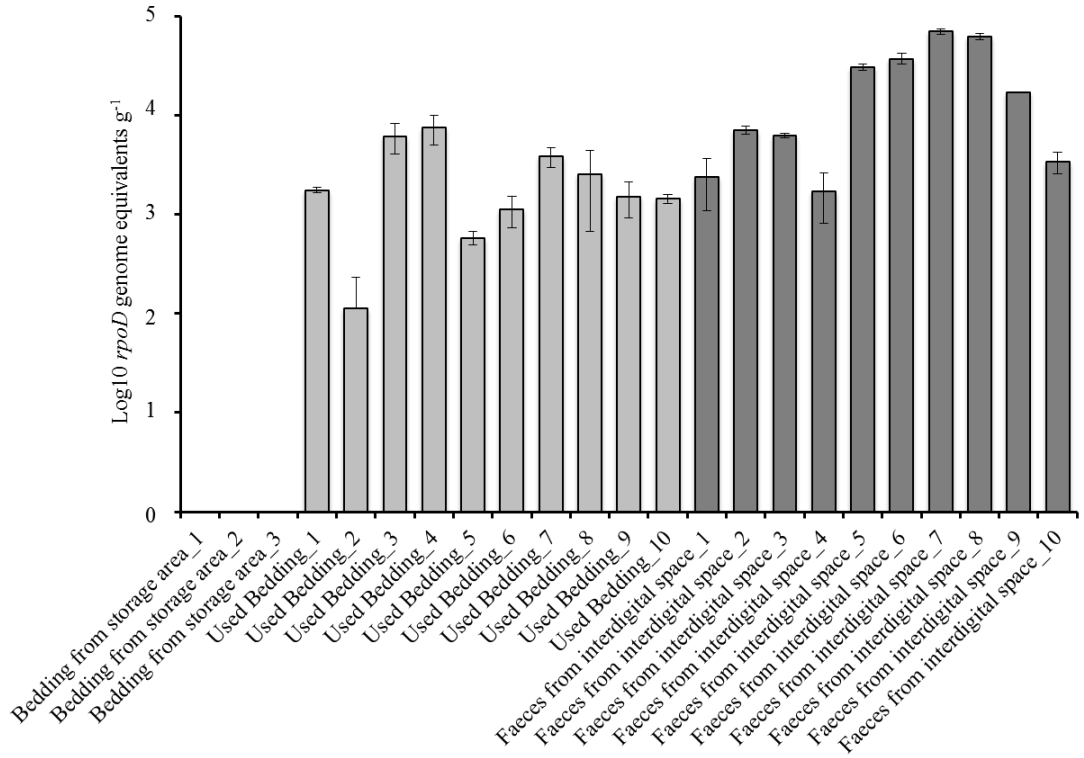


Figure 2.1. Presence of *D. nodosus* in the environmental samples. Absolute quantification of *rpoD* gene in bedding samples from the storage area, used bedding samples and faeces compacted within the interdigital space. Each bar is the average of triplicate analyses, error bars represent ± standard deviation.

2.4.2. Quantification of *D*. *nodosus* in ewes and lambs.

D. *nodosus* was not detected in lambs' feet at birth but was detected in all lambs and

ewes 5-13 hours later after their feet had touched the floor initially in a large communal pen, and subsequently in an individual pen. *D. nodosus* was detected on 39 / 40 (97.5%) of ewes' feet and 39 / 40 (97.5%) of lambs' feet (Figure 2.2). Whilst overall the population loads were significantly higher in ewes than lambs (Mann Whitney U test; p-value < 0.001); analysis of ewe / lamb pairs suggested only ewes 2, 3 and 5 had a higher load than their lambs. The *D. nodosus* load ranged from 10^3 to 10^5 *rpoD* genome equivalents per swab in lambs and 10^2 to 10^7 *rpoD* genome equivalents per swab in ewes (Figure 2.3).

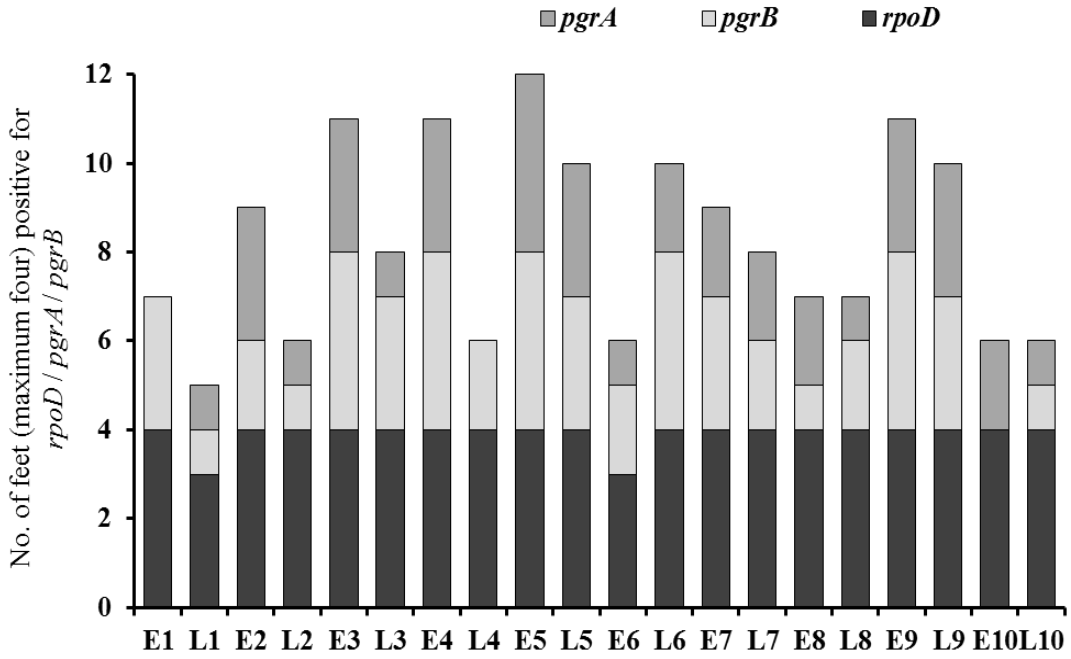


Figure 2.2. Detection of *pgr* variants in the community DNA. Presence of *pgrA*, *pgrB* and *rpoD* on the feet of 10 ewes and 10 lambs. *pgrA* / *B* was absent/below the detection limit in the samples where no data is shown. (E = Ewe; L = Lamb).

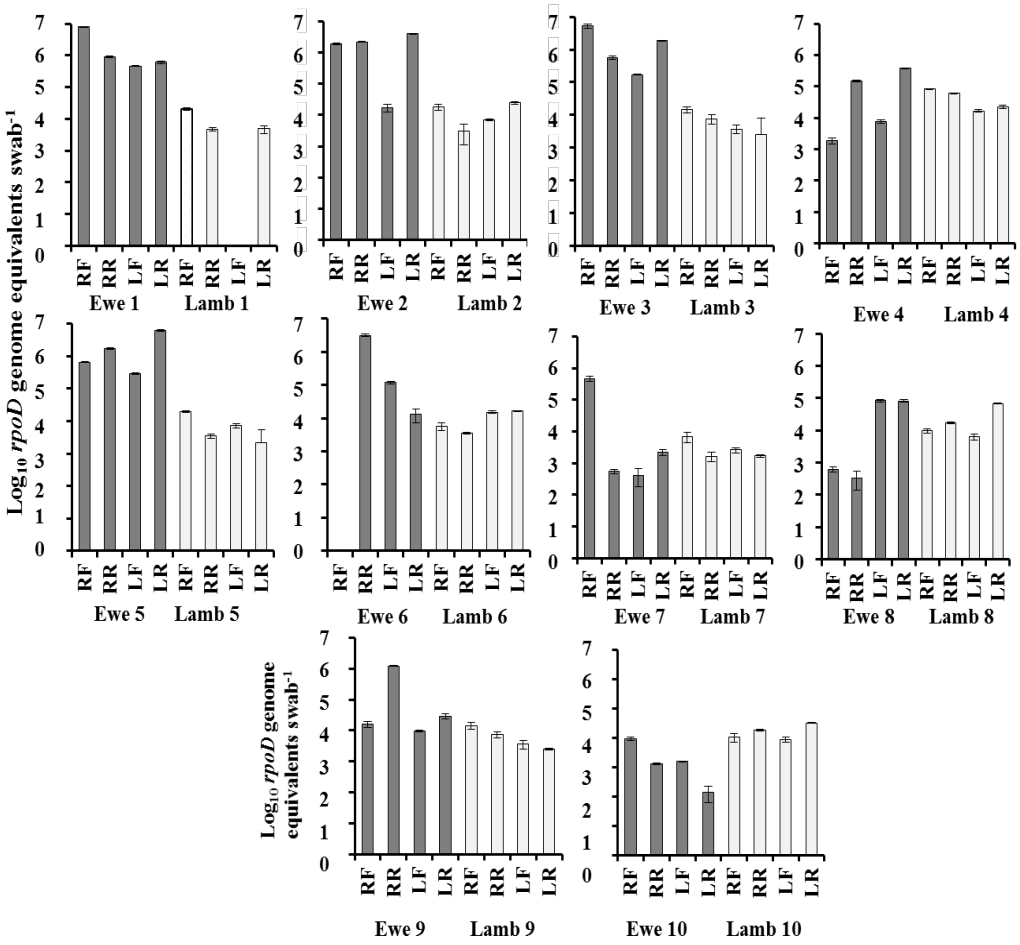


Figure 2.3. Enumeration of *D. nodosus* by *rpoD* qPCR. Approximate measure of *D*. *nodosus* abundance estimated by a qPCR assay for *rpoD*. Overall, *D*. *nodosus* was significantly higher in ewes than their lambs (p-value < 0.001). (Error bars represent ± standard deviation).

2.4.3. Detection of *pgr* variants in the community DNA.

pgrA was detected on 23 / 40 (57.5%) of ewes' feet and 15 / 40 (37.5%) of lambs' feet, whereas *pgrB* was detected on 27 / 40 (67.5%) of ewes' feet and 22 / 40 (55%) of lambs' feet (Figure 2.2). Both variants *pgrA* and *pgrB* were detected on eight ewes and nine lambs.

Forty-two *pgrA* clones were sequenced from 14 foot swabs from 5 ewes and 10 foot swabs from 5 lambs. This resulted in the detection of 11 variants containing 3 - 21

tandem repeats in the R1 region, with 2 - 6 variants per animal (Table 2.5). Multiple *pgrA* variants with varying numbers of tandem repeats were observed in a single foot swab (Table 2.6)

Table 2.5. Distribution of *pgrA* R1 tandem repeats in five pairs of ewes and lambs (14 ewe and 10 lamb feet) E = Ewe and L=Lamb.

Ewe/Lamb ID	Number of clones sequenced	Number of *pgrA* tandem repeats in the R1 region
E 1	6	3, 4, 5, 11, 13, 16
L 1	4	4, 11, 12, 16
E 2	3	4, 11, 15
L 2	5	3, 4, 6, 11, 16
E 3	4	6, 15, 16, 21
L 3	2	16, 20
E 4	4	4, 6, 11, 13
L 4	5	4, 6, 11, 12, 16
E 5	4	5, 12, 15, 16
L 5	5	4, 11, 12, 15, 16

Table 2.6. Distribution of *pgrA* R1 tandem repeats in five pairs of ewes and lambs (data presented at foot level). E = Ewe, L = Lamb.

Ewe/Lamb	Right front	Right hind	Left front	Left hind
E1	3, 4	5	11	13, 16
L1	4	-	12	11, 16
E2	11	4	15	-
L2	3, 11	4, 6	16	-
E3	6, 16	16, 20	-	-
L3	16	-	-	20
E4	-	-	6, 11	4, 13
L4	-	-	4, 6, 11, 12, 16	-
E5	12, 15	-	5	16
L5	4, 11, 12, 15, 16	-	-	-

2.4.4. Molecular typing MLVA from the community DNA.

Alleles at *D. nodosus* tandem repeat DNTR19 were detected on the feet of six ewes and their lambs whereas for DNTR10 alleles were only detected on two ewe / lamb pairs (Figure 2.4, Table 2.7). The fluorescent data for DNTR09 was below the peak height threshold level (40 fluorescence units) and DNTR02 primers demonstrated some non-specific binding, so were excluded from the analysis. For both loci (DNTR10 and DNTR19), one or two alleles occurred in lambs, but there was greater diversity in ewes, with up to six alleles detected (Table 2.7). As detectable diversity in ewes increased, so did the likelihood of detecting the same strain on its offspring. The overall coincidence index of overlap between ewes and lambs was 0.45.

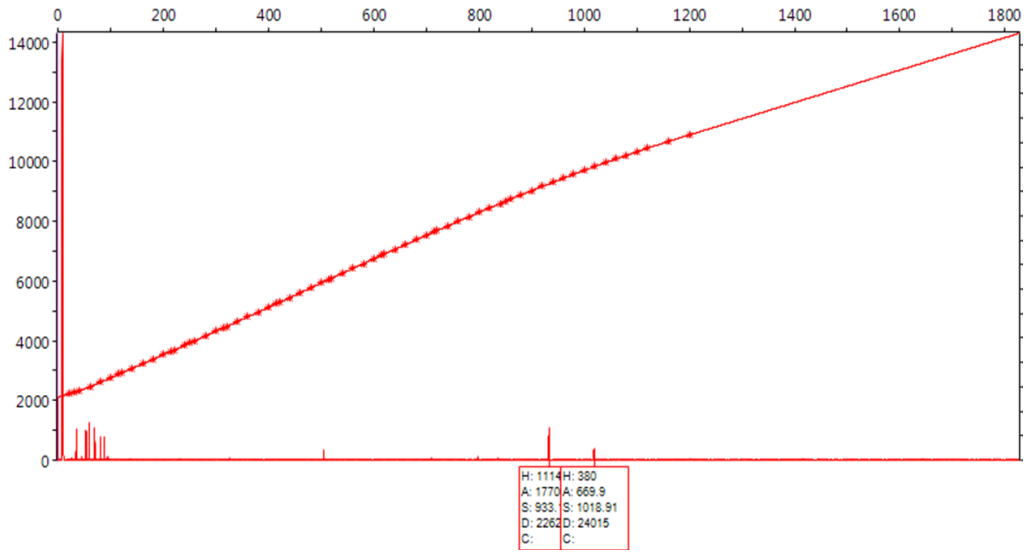


Figure 2.4. Presence of DNTR19 alleles in Ewe 1. DNTR19 alleles were present on the left front foot in Ewe 1. The size of the alleles is 933.18 and 1018.91 base pairs that correspond to 5 and 6 tandem repeats respectively. The additional peaks in the figure were not analysed due to their occurrence before the cut off size of 500 bp. The asterisk on the slope are different size fragments of GeneScan 1200 LIZ dye size standard.

Table 2.7. DNTR19 and DNTR10 allelic distribution between six and two pairs of ewes and lambs.

ID	DNTR19							DNTR10					
	3+	4	5	6	7	8	Total	3	4	7	9	10	Total
E 1	1	1	1	1	1	1	6	1	1	1	1	1	5
L 1	0	0	0	1	0	0	1	1	0	0	0	0	1
E 2	1	1	0	1	0	0	3	1	0	0	1	0	2
L 2	1	0	0	0	0	0	1	1	0	0	0	0	1
E 3	0	1	0	0	0	0	1						
L 3	1	0	0	0	0	0	1						
E 4	0	1	1	0	0	0	2						
L 4	0	0	0	1	0	0	1						
E 5	1	1	1	1	1	0	5						
L 5	0	0	0	0	1	0	1						
E 6	0	1	0	0	0	0	1						
L 6	0	0	0	0	0	1	1						

+ (The numbers given in the table heading are the number of tandem repeats).

2.5. Discussion

In the current study we demonstrated, for the first time, that lambs are born free from *D*. *nodosus* (given the sensitivity of the tests used) but that we were able to detect high levels of *D*. *nodosus* on foot swabs within a few hours of birth. The most likely route of transmission is from standing on contaminated bedding in communal pens, however, recent work (Witcomb, 2012) indicated that *D*. *nodosus* can be detected in the mouths of ewes, so transmission might also have occurred as the lamb was being cleaned by the ewe after birth. Quantitative PCR with specific primers is widely used to determine bacterial load from swabs (Fredricks et al., 2009; Jaton et al., 2006; Lund et al., 2004) when studying disease development over time (Srinivasan et al., 2010). Here, as elsewhere (Witcomb et al., 2014) qPCR of *rpoD* was used to determine *D*. *nodosus* load on the feet of ewes and lambs. It was striking that such a large *D*. *nodosus* population was present on the feet of lambs within a few hours of birth. This suggests that *D*. *nodosus* might be an early coloniser of lambs' feet, although, it could be that naïve feet are simply colonized by the first bacterial species they encounter.

Several serogroups of *D*. *nodosus* on individual feet have been reported elsewhere (Claxton et al., 1983; Hindmarsh and Fraser, 1985; Jelinek et al., 2000; Moore et al., 2005a), but not using the typing methods used in the current study where both *pgr* data and MLVA provided evidence for the occurrence of multiple strains in most animals studied. Not all the *D*. *nodosus* strains detected on the feet of lambs were on their mother's feet, indicating that the strains detected on lambs' feet originated from sources additional to their mothers' feet, most likely contaminated bedding in the communal

pen. In addition, not all the strains detected in ewes were present in their offspring. It is possible that the specific variants on the ewes were not detected by chance, or were present below the minimum detection level or that only some of the strains were transferred via bedding.

Given the survival time of *D. nodosus* off host and the *D. nodosus*-negative stored straw samples, the most probable source of *D. nodosus* was the population of ewes in the communal pen. Methods to reduce the load of *D. nodosus* in ewes and the environment would probably have the biggest impact on transmission to newborn lambs. Management strategies that have been linked to a reduction in footrot prevalence and incidence include rapid appropriate treatment of diseased sheep (Kaler et al., 2010b; Wassink et al., 2010b), segregation of diseased sheep and footbathing healthy sheep (Wassink et al., 2004).

The identification of transmission routes and understanding the role of the environment is critical for the control of footrot. Previous studies have highlighted that diseased sheep are a reservoir of infection (Green et al., 2007; Kaler et al., 2010b; Smith et al., 2014), although transmission occurs indirectly via contaminated pasture or floors (Beveridge, 1941). The current study has demonstrated that the environment potentially forms at least a temporary reservoir of infection for lambs because *D. nodosus* was detected in the straw bedding in the communal pens, and lambs have strains of *D. nodosus* on their feet not detected on their mother's feet.

The fact that lambs were *D*. *nodosus*-negative at birth suggests that it might be possible to produce *D*. *nodosus*-free lambs without the need for a caesarean birth. This finding is of interest to those performing challenge studies on pathogen-free individuals. At the practical, farm level this information might also be useful in countries that have eradicated footrot. In countries where footrot is endemic, even if lambs could be kept *D*. *nodosus*-free around lambing time, it is highly unlikely that this status could be maintained because of the high levels of infection in ewes. Reducing initial exposure (as suggested above) might be beneficial to subsequent disease severity, however, it might be detrimental if later age at first exposure increases disease severity.

2.6. Conclusion.

We have provided evidence that lambs are born *D*. *nodosus*-negative, but within hours of birth several strains of *D*. *nodosus* are detectable on their feet. The strains detected were a combination of those present on their mother's feet and on the feet of other ewes. Straw bedding in the communal pen was *D*. *nodosus*-positive and the most likely source of *D*. *nodosus* for newborn lambs.

CHAPTER 3

Survival of the ovine footrot pathogen *Dichelobacter nodosus* in different soils.

3.1. Abstract.

Dichelobacter nodosus (*D*. *nodosus*) is the causative agent of footrot in sheep; one of the most important health and welfare issues of sheep worldwide. For control programmes to be effective, it is essential that the transmission cycle of *D*. *nodosus* is understood and bacterial reservoirs in the environment are better defined. This study evaluated the survival of *D*. *nodosus* in different soils using soil microcosms. Cultivation independent and dependent methods were used to detect *D*. *nodosus* over 40 days from seeding in soil. A *D*. *nodosus* specific probe was used for quantification by qPCR and viability was assessed by cell permeability to an intercalating dye, PMA, and by culture. Survival varied dramatically depending on soil type, matric potential (MP) and temperature. Our findings indicate that *D*. *nodosus* survival was higher at 5 ^{o}C compared with 25 ^{o}C in all soils and significantly longer at both temperatures in clay soil (>44% clay) compared with other soil types. Survival under all conditions was longer than 30 days for both culture independent and dependent methods, this is substantially longer than reported in previous studies and, if this is an infectious dose, longer than the current recommendation of resting a field for 14 days to prevent onward infection.

3.2. Introduction.

Ovine footrot is an infectious disease of sheep that is caused by an anaerobic bacterium *Dichelobacter nodosus*, which is the essential transmitting agent (Kennan et al., 2001; Kennan et al., 2010; Witcomb et al., 2014). The disease spreads between sheep via pasture or bedding. It is characterized by interdigital inflammation, with or without separation of the keratinous hoof horn from the underlying dermis. Both conditions result in lameness and loss of body condition (Egerton et al., 1969; Stewart, 1989; Wassink et al., 2010a).

Footrot is the main cause of lameness in sheep in the UK (Grogono-Thomas and Johnston, 1997; Kaler and Green, 2008) and accounts for serious economic losses in countries worldwide (Green and George, 2008; Stewart, 1989; Wani and Samanta, 2006). Recent reports indicate that footrot results in annual losses of between £24 and £80 million in the UK (Nieuwhof and Bishop, 2005; Wassink et al., 2010a). Its significant financial impact is due to a reduction in meat and wool production and the expenditure associated with treatment and prevention (Green and George, 2008; Stewart, 1989; Wani and Samanta, 2006; Wassink et al., 2010a). It is a painful condition and thus is an important and challenging welfare issue (Fitzpatrick et al., 2006).

Footrot is seasonal in some areas of the world, particularly arid areas of Australia and India, where there are predictable periods of transmission (Green and George, 2008). In the UK, there is no seasonality for severe footrot, rather a series of mini-epidemics throughout the year (Green et al., 2007) with increasing temperature and rainfall

favouring spread of the disease (Smith et al., 2014). Epidemics of interdigital dermatitis are also reported in spring (Wassink et al., 2003).

Part of the strategy used in control and elimination programmes is empirical evidence that *D. nodosus* cannot survive for more than a few days off the feet of ruminants (Abbott and Lewis, 2005; Beveridge, 1941). Survival away from the host is dependent on moist, mild conditions (Graham and Egerton, 1968) and *D. nodosus* is reported to survive at an infectious dose for no longer than two weeks on pasture under optimal (warm and damp) conditions (Beveridge, 1941; Whittington, 1995). A recent study using qPCR demonstrated that *D. nodosus* can survive up to 14 days in soil microcosms at 5 ^{o}C and for a further 24 days if powdered hoof horn was added to soil; survival was markedly reduced at 15 ^{o}C however, the moisture content was not stated (Cederlof et al., 2013). Nevertheless such work is important to move to an evidence-based approach to manage environmental contamination with *D. nodosus*.

To date, the environmental conditions and soil types where survival is greater, have not been elucidated and further work is warranted on *D. nodosus* survival outside the host, taking into account edaphic factors that result in dramatic differences in matric potential and water availability. Whilst a large number of studies have focused on *E. coli* survival in soil, it is still uncertain how fluctuations in water availability affect survival (van Elsas et al., 2011). Survival studies are uninformative without taking soil type into account if bulk soil is used in microcosms. For these reasons this study focused on the

survival of *D*. *nodosus* in soil to investigate the impact of soil type, temperature and matric potential on longevity of viable *D*. *nodosus* in soil.

3.3. Material and Methods

3.3.1. Soil desiccation curves.

To select the appropriate microcosm set up, drying out of soil was determined for up to 17 days using Warwick soil. Four different microcosm types were set up using 20 g soil in 50 ml falcon tube and included closed, open, partially closed and closed with aeration vents (two holes punched in lid and two in sides above soil level). Soil was prepared as previously reported (Young et al., 2005). Soil samples were moistened to approx.-33 kPa (18% water). The microcosms were incubated at 25 °C in the dark and reduction in weight was recorded at each day until reductions in weights were constant.

3.3.2. Soil microcosms.

Soil microcosms (50 ml Falcon tubes without aeration vents) containing 5 g of non-sterile Warwick soil (sandy loam) were set up in triplicate at 18% water content (-33kPa, matric potential (MP defined as the suction required to extract water as soils dry out (Artz et al., 2006)) calculated from previous wetting-drying curves of Warwick soil El-Muntaser MSc Thesis 2009; Figure. 3.1). *D*. *nodosus* was inoculated to the soil microcosms to a final concentration of 10^8 cells g^{-1} soil at 18% moisture content. Microcosms were incubated aerobically at 25 °C in the dark and destructive sampling was performed at days 0, 1, 2, 3, 6, 10, 14, and 21. A sample of soil 0.5 g was taken for

DNA extraction and 0.5 g for RNA extraction to which 1 ml of RNA*later* Stabilization Solution (Life Technologies Ltd, Paisley, UK) was added and stored at -80 °C.

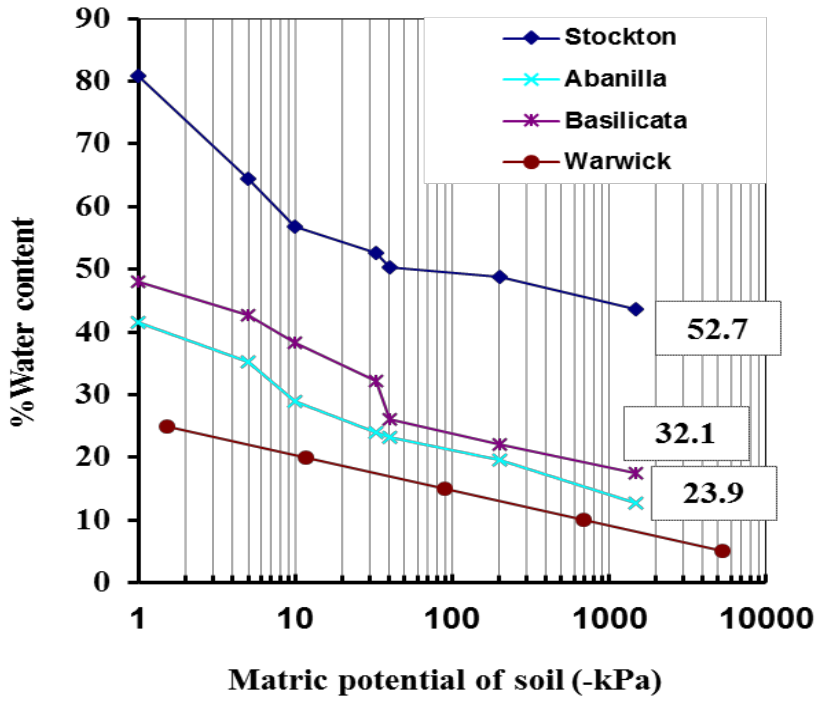


Figure 3.1. Relationship between water content and matric potential (MP) in four soil types. The final figure in the box denotes moisture (-33 kPa) MP of soil.

Soil microcosms with different non-sterile soil types (20 g) including Warwick (sandy loam), Basilicata (sandy loam), Abanilla (sandy) and Stockton (clay) (soil characteristics Table 3.1; wetting-drying curves Fig. 3.1) were set up in triplicate, inoculated with *D*. *nodosus* culture (10^7 cells g^{-1} soil) and incubated at 25 °C in the dark. Further, a second triplicate set of microcosms were incubated at 5 °C and destructive sampling of both sets was performed at days 0, 3, 6, 9, 12, 20, 30, and 40. Uninoculated

soil was used as control to determine *D. nodosus* background amplification. Soils types Basilicata, Abanilla, Stockton and Warwick were collected from Italy, Spain and Scotland and England respectively from 1 square foot area of land. These soils were collected in bulk in the plastic bags and brought to the laboratory for storage. The soils were processed in a similar manner as Warwick soil using the protocol as previously reported (Young et al., 2005).

Table 3.1: Characteristics of four soil types.

Soil type	pH	Sand	Silt	Clay	TOC	Moisture	USDA texture
Stockton	8.00	17.65	37.93	44.42	10.89	52.7	Clay
Abanilla	7.39	63.60	11.52	28.32	1.00	23.9	Sandy Loam
Basilicata	8.16	60.00	11.76	27.12	1.39	32.1	Sandy Loam
Warwick	8.00	63.00	18.40	11.70	6.2	18.0	Sandy Loam

3.3.3. Cultivation of *D. nodosus* on solid and liquid media and its growth conditions

A virulent strain of *D. nodosus* (VCS1703A) was cultured on 2-4% Trypticase Arginine Serine Hoof agar (TASH) and Trypticase Arginine Serine (TAS) (Becton, Sparks Maryland, USA; Sigma-Aldrich, St. Louis, USA) and incubated at 37 ^{o}C for 3-4 days in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, UK) (Skerman, 1975; Thomas, 1958) (Appendix 3). The cells were harvested in 2 ml phosphate buffer saline (PBS) solution to yield a final concentration of approximately 10^{8} cells ml^{-1}.

3.3.4. Extraction of DNA from soil.

DNA was extracted from 0.5 g soil samples using the FastDNA Spin kit for Soil following the manufacturer's instructions (MP Biomedicals, UK). DNA was then eluted into 70 µl of DNase Free water. Sterile soil was included as blank.

3.3.5. Extraction of RNA and synthesis of cDNA.

RNA was extracted from Warwick soil samples collected at different time points using RNeasy Mini Kit (QIAGEN, Manchester, UK) and Griffith's method with some modifications. RNA integrity and concentration were measured using the Prokaryote Total RNA nano (Agilent Bioanalyser; Appendix 4). DNase treatment of the total nucleic acid was performed using Turbo DNA free kit following the manufacturer's instructions. From DNA-free RNA, cDNA was synthesized using High capacity RNA to-cDNA Kit (Applied Biosystems, Warrington, UK).

3.3.6. End point PCR and qPCR.

PCR amplifications targeting *D. nodosus* 16S rRNA were performed using specific primers Cc and Ac (La Fontaine et al., 1993) in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) as described previously (Muzafar et al., 2015) (Table 3.2). *D. nodosus* DNA from the strain, VCS1703A was included as a positive control and sterile water as a negative control.

Table 3.2. Primers and Taqman probe used in the study

Primer name	Sequence (5'- 3')
D. nodosus 16S *rRNA* Forward (Cc)	CGGGGTTATGTAGCTTGC
D. nodosus 16S *rRNA* Reverse (Ac)	TCGGTACCGAGTATTTCTACCCAACAC
rpoD Forward	CTGCTCCCATTTCGCGCATAT
rpoD Reverse	CTGATGCAGAAGTCGGTAGAACA
Taqman *rpoD*	(6FAM)-CATTCTTACCGGKCG-BBQ2

The qPCR of the DNA and cDNA was performed in an Applied Biosystems, 7500 Fast real-time detection system (Applied Biosystems, Warrington, UK). The PCR targeted the *rpoD* gene (RNA polymerase sigma 70 factor, single copy number in *D. nodosus* genome) (Calvo-Bado et al., 2011a). All PCR reactions were performed in triplicate as previously reported (Muzafar et al., 2015). The *rpoD* copy number was estimated based on the standard curve obtained from analysis of ten-fold serial dilutions of DNA extracted from *D. nodosus* strain VCS1703A. The results were analyzed using 7500 Fast System SDS software (Applied Biosystems, Warrington, UK).

3.3.7. Cultivation of *D. nodosus* from soil microcosms.

Soil samples taken at different time points over 40 days from the inoculated microcosms were streaked (approximately 100 µg attached to the tip of the loop and soil suspension (1:10) in quarter strength Ringer's (100 µl)) on 2% TASH plates and incubated anaerobically at 37 ^{o}C for 3-4 days. The colonies on 2% TASH plates were sub cultured on 4% TASH and again incubated anaerobically at 37 ^{o}C for 3-4 days for isolating the pure colonies. The colonies obtained on the 4% TASH were confirmed as *D. nodosus* by Gram staining and a *D. nodosus* specific 16S rRNA gene colony PCR.

3.3.8. Live and dead cell microscopy and propidium monoazide treatment.

The presence of live and dead cells in *D. nodosus* culture (cells were heat treated at 100 ^{o}C for 15 minutes to obtain dead cells while untreated served as live cells) was analyzed using 20 mM DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) and PI (Propidium Iodide) incorporation assay. *D. nodosus* cells were washed and resuspended in 0.85 NaCl, to which a mixture of DAPI and PI was added and incubated for 15 min in the dark. The cells were observed under fluorescent microscope to confirm the dead cells.

Anaerobically cultured *D. nodosus* cells on 2% TASH were harvested (10^6 cells ml^{-1}) and heat treated at 100 ^{o}C for 15 min to produce dead cells. Untreated (live cells) served as a control. Serial dilutions of *D. nodosus* live and dead cells starting from 10^6 ml^{-1} were treated with propidium monoazide (PMA; Biotium, Hayward, CA) by following the manufacturer's instructions with some modifications. 7 µl of PMA was added to 500 µl cell suspension and incubated in the dark for 20 minutes followed by a light treatment for 10 minutes that was carried on ice. During light treatment, the transparent eppendorf tubes were occasionally rotated to ensure that whole culture is exposed to the light. PMA penetrates the compromised membranes of dead cells and intercalates with the DNA, inhibiting PCR amplification (Nocker et al., 2009). DNA was extracted from non-PMA treated live cells and PMA treated dead cells using DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK) following the manufacturer's instructions. Quantification of *D. nodosus* was determined by a qPCR assay targeting *rpoD* gene.

Soil samples taken at different time points from Warwick soil microcosms were PMA treated as described above with some modifications. 10 µl of PMA was added to 500 µl soil suspension (0.5 g soil dissolved in 500 µl water) and incubated in the dark for 20 minutes followed by a light treatment for 20 minutes with occasional as described above. DNA was extracted from PMA treated samples using the FastDNA Spin kit for Soil (MP Biomedicals, UK) by following the manufacturer's instructions. A qPCR assay targeting *rpoD* was performed to determine the number of live cells in the given sample.

3.3.9. Turnover of *D. nodosus* DNA in soil.

Soil microcosms containing 1 g Warwick soil were inoculated with *D. nodosus* in the form of live cells, dead but intact (UV treated), dead (Heat killed) and free DNA at a final concentration of 10^7 cells and *rpoD* genome equivalents in case of free DNA. The microcosms were moistened to -33 kPa (18% moisture) and incubated at 25 ^{o}C in the dark. Destructive sampling was performed in triplicate at days 0, 4, 7, 10, 14, 20, 25, 30, 40, 50 and 60. DNA was extracted from 0.5 g soil and *D. nodosus* was enumerated by a qPCR assay targeting *rpoD* gene.

3.3.10. Statistical analysis.

The *rpoD* mean counts obtained from the qPCR were compared between test days for Warwick soil using Student's T-Test in SPSS to test for significant differences in the amount of *D. nodosus* detected at various time points. The exponential decay rates were calculated using the formula $P(t) = P_0 e^{-kt}$, where P (t) = Amount of *D. nodosus rpoD*

genome equivalents at time t; P_0 = Initial amount of *D*. *nodosus rpoD* genome equivalents at time t = 0; k = the decay rate; t = time (number of periods). Models were built using MlwiN 2.1 (Rasbash et al., 2009) to investigate soil parameters including soil type, incubation time, temperature, pH, total organic carbon, moisture, sand, silt, clay in univariable and multivariable mixed effect models to determine whether all parameters together or individually predicted the survival of *D*. *nodosus* in soil.

3.4. Results.

3.4.1. Impact of aeration and survival of *D*. *nodosus* by soil type and temperature.

For the first 10 days, moisture declined most rapidly in the open microcosms at 0.015 g day^{-1} followed by ventilated, (0.014 g day^{-1}) partially closed, (0.002 g day^{-1}) and closed microcosms, (0.0001 g day^{-1}) (Figure 3.3); after 10 days rates were constant. Therefore, the microcosm with aeration vents was chosen for further experiments.

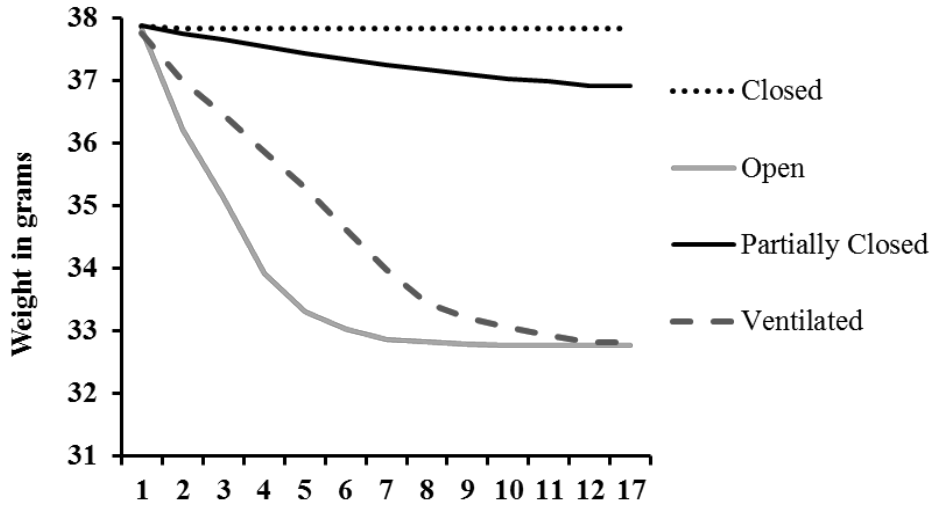


Figure 3.2. Soil desiccation curves. Drying out of soil under different microcosm types, closed; partially closed; ventilated; open. Drying out of soil was determined using 20 g of Warwick soil using four different microcosm setups. The decline in moisture was determined at each time point until the complete drying out had occurred.

D. *nodosus* 16S rDNA was detected by end point PCR in all soil types at each time point during the 40-day study at both 5 ^{o}C and 25 ^{o}C. *D*. *nodosus* colonies were observed in culture over 40 days and confirmed by staining and colony PCR with the exception of Warwick soil at 25 ^{o}C on day 40 (Table 3.3, Appendix 5).

A biphasic rate of decay in *D*. *nodosus* populations in the four soil types was observed (Table 3.4; Figure 3.3); Stockton (heavy clay) had a significantly slower rate of decay in both phases compared with other soil types. In the univariable mixed effects model, the decay in survival over time of genome equivalents of *D*. *nodosus* was a cubic function of day (days^1, ^2 and ^3) demonstrating a non-linear decay over time. The %TOC, %moisture, %clay, %silt, were significantly associated with increased survival time,

%sand was associated with a decreased survival time and pH was not associated with survival time when each of these variables were fitted in turn (Table 3.5).

In the multivariable model, once survival over time was fitted, *D*. *nodosus* survived for significantly longer at 5 ^{o}C compared with 25 ^{o}C and in Stockton (heavy clay) soil compared with other soil types (Table 3.6). No other variables were significant because soil types were highly associated to %TOC, moisture, %clay, % silt (Table 3.1).

Table 3.3. Detection of viable cells using cultivation on hoof horn medium. Colonies confirmed by *D. nodosus* 16S rRNA gene colony PCR (3 replicates).

Time point	Warwick 25 °C	Warwick 5 °C	Abanilla 25 °C	Abanilla 5 °C	Basilicata 25 °C	Basilicata 5 °C	Stockton 25 °C	Stockton 5 °C
Day 0	+++	+++	+++	+++	+++	+++	+++	+++
Day 3	+++	+++	+++	+++	+++	+++	+++	+++
Day 6	+++	+++	+++	+++	+++	+++	+++	+++
Day 9	+++	+++	+++	+++	+++	+++	+++	+++
Day 12	+++	+++	+++	+++	+++	+++	+++	+++
Day 20	+++	+++	+++	+++	+++	+++	+++	+++
Day 30	+++	+++	+++	+++	+++	+++	+++	+++
Day 40	---	+++	+++	+++	+++	+++	+++	+++

Table 3.4. Rate of decline per day of *D. nodosus* in four different soil types at two temperatures over two phases.

Soil Type	Decay rates at 25 ^{o}C (DNA decrease k day^{-1})		Decay rates at 5 ^{o}C (DNA decrease k day^{-1})	
	(D0 – D9)	(D9 – D40)	D0 – D30	D30 – D40
Warwick (Sandy Loam)	0.96	0.56	0.84	0.94
Basilicata (Silt)	0.96	0.67	0.88	0.96
Abanilla (More sandy)	0.95	0.49	0.52	0.94
Stockton (Clay)	0.61	0.78	0.60	0.28

'D' denotes time interval in days for rate calculated

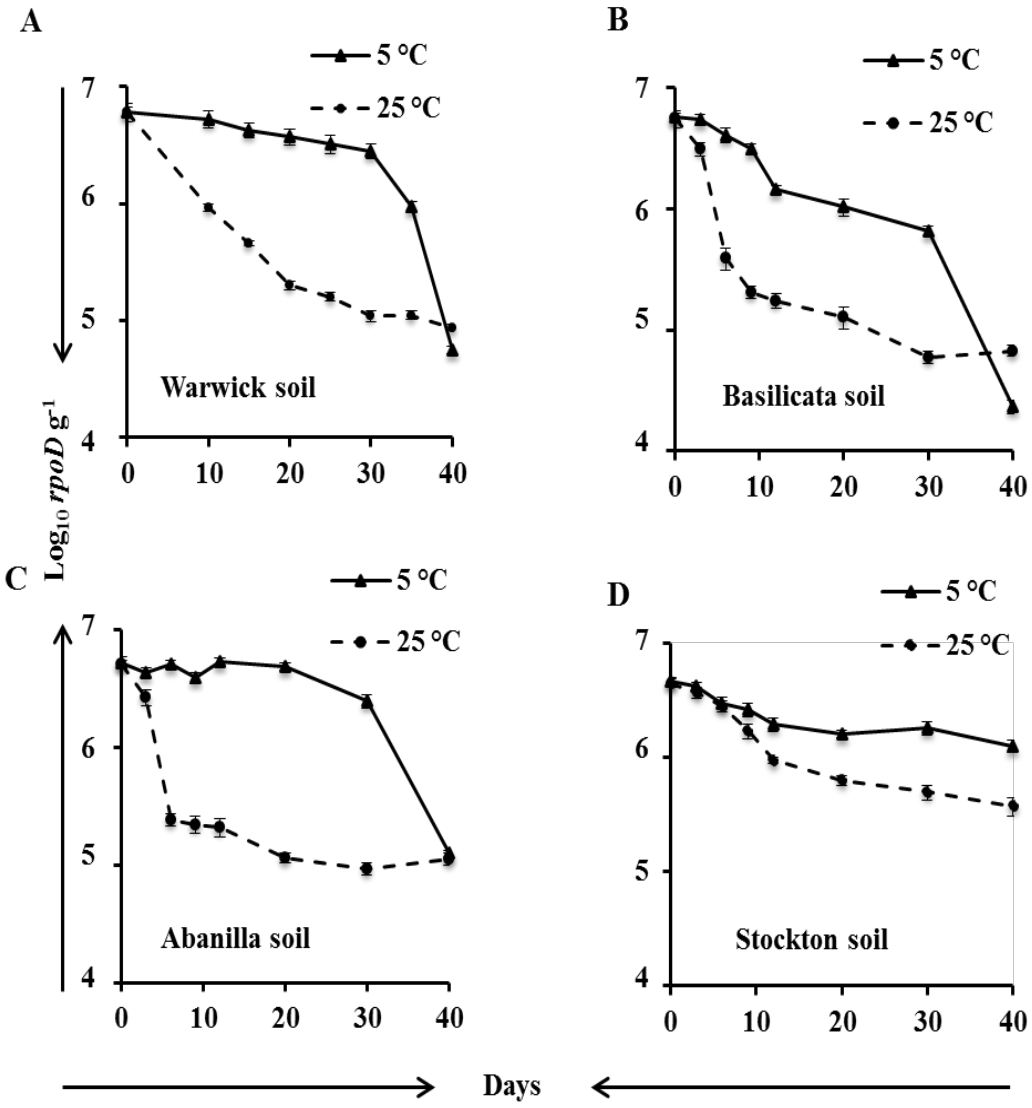


Figure 3.3. Absolute quantification of *rpoD* gene from DNA over 40 days (Error bars represent ± SD). **(A)** Quantification of *rpoD* gene from Warwick soil. At day 0, *D*. *nodosus* was present at 10^7 copies and declined to 10^5 over 40 days. **(B)** Quantification of *rpoD* gene from Basilicata soil. **(C)** Quantification of *rpoD* gene from Abanilla soil. **(D)** Quantification of *rpoD* gene from the Stockton soil. The DNA was extracted using fast DNA spin kit for soil and elution was done in 70 µl of the elution buffer.

Table 3.5. Univariable mixed effect models for associations between genome equivalents of *D.nodosus* and different variables.

Variable	Mean	S.E	Lower 95% CI	Upper 95% CI
Intercept	7.756	1.225	5.355	10.157
Days^1	-0.116	0.028	-0.171	-0.061
Days^2	0.005	0.002	0.001	0.009
Days^3	0.000	0.000	0.000	0.000
Temp 5 ^{o}C	baseline			
Temp 25 ^{o}C	-0.647	0.091	-0.825	-0.469
pH	-0.086	0.155	-0.390	0.218
Sand%	-0.007	0.002	-0.011	-0.003
Silt%	0.013	0.004	0.005	0.021
Clay%	0.011	0.004	0.003	0.019
TOC%	0.031	0.011	0.009	0.053
Moisture%	0.010	0.003	0.004	0.016
Clay soil	baseline			
Soil type_1	-0.35	0.116	-0.577	-0.123
Soil type_2	-0.431	0.116	-0.658	-0.204
Soil type_3	-0.254	0.116	-0.481	-0.027

^ = to the power of. S.E = Standard Error.

Table 3.6. Mixed effects model of factors associated with mean *D. nodosus* genome equivalents adjusted for replicate and day, 192 sample.

Variable	Mean	S.E.	Lower 95% CI	Upper 95% CI
Intercept	6.987	0.129	6.734	7.240
Days^1	-0.116	0.025	-0.165	-0.067
Days^2	0.005	0.002	0.001	0.009
Days^3	0.000	0.000	0.000	0.000
Temp 5^{o}C	baseline			
Temp 25^{o}C	-0.647	0.082	-0.808	-0.486
Clay soil	baseline			
Soil type_1	-0.35	0.116	-0.577	-0.123
Soil type_2	-0.431	0.116	-0.658	-0.204
Soil type_3	-0.254	0.116	-0.481	-0.027

3.4.2. Persistence of metabolically active cells in the soil microcosms.

Survival studies indicated that *D. nodosus* had a biphasic decline in soil with a rate of 0.53 k day $^{-1}$ up to day 6 and 0.12 k day $^{-1}$ from 7-14 days, these rates are significantly different ($P<0.05$) (Figure 3.4A). Metabolically active cells were detected using cDNA from *rpoD* RNA and declined in a biphasic manner as observed for DNA genome equivalents at 0.517 k day $^{-1}$ from day 1-6 and 0.021 k day $^{-1}$ from day 6-21 which was significantly lower than the initial rate of decline ($p<0.001$); the decay rate was constant after day 6 (Figure 3.4B).

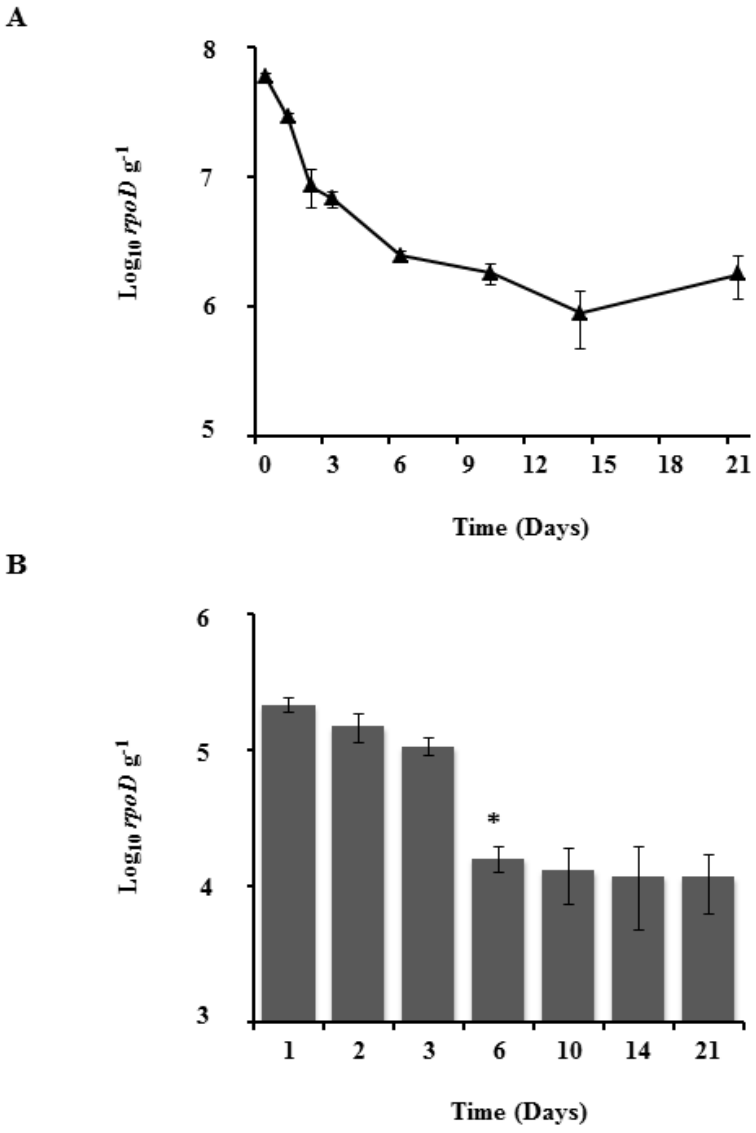


Figure 3.4. Molecular detection and quantification of *D. nodosus* in soil. Bars represent mean ± SD; n=3; *p<0.001. (A) Quantification of *D. nodosus* by qPCR targeting *rpoD* gene **(B)** Detection of metabolically active cells using cDNA qPCR derived from RNA.

3.4.3. Direct evidence for survival of viable cells in Warwick soil microcosms.

The heat-treated cells were dead as observed by PI staining (Figure 3.5A). PMA treatment of dead cells resulted in a significant reduction in amplification (Figure 3.5B) with background amplification of 10^2 cells, which was constant throughout the dilution series. These results confirm that PMA detected dead cells. At day 30, *D. nodosus* was present in Warwick soil at 10^5 *rpoD* genome equivalents as detected by qPCR but PMA treatment led to significant reduction ($p < 0.001$) to just above the detection limit of 10^2 *rpoD* genome equivalents. There was no significant difference in the number of live cells observed at 5 ^oC and 25 ^oC at day 30 (Figure 3.5C).

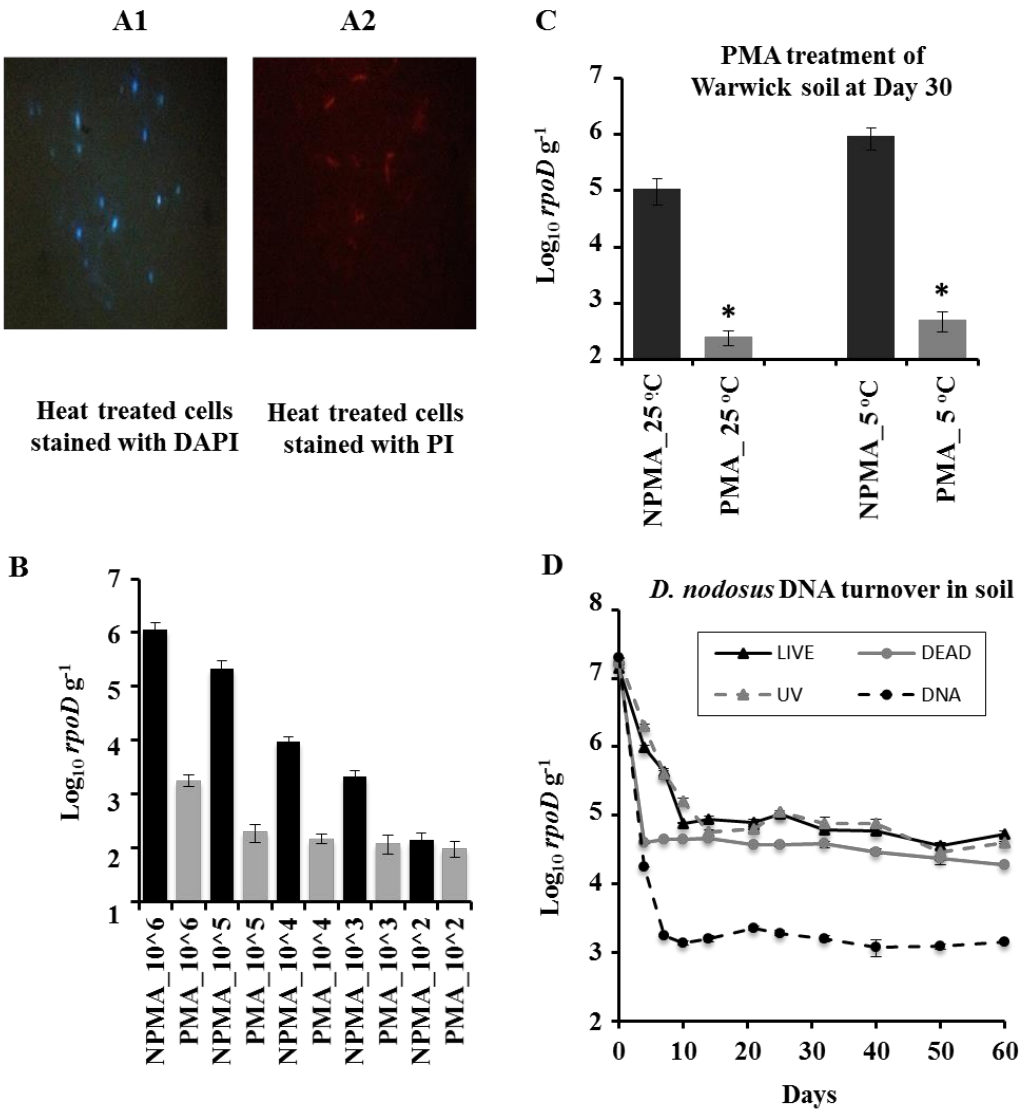


Figure 3.5. Viability of *D. nodosus* using propidium monoazide method. **(A1)** DAPI stained heat treated cells. **(A2)** Live-dead staining with PI, red indicating dead cells. **(B)** Quantification of *rpoD* gene of the PMA treated *D. nodosus* cells at various dilutions. PMA denoted PMA treated and non-treated is NPMA. **(C)** Comparison of non-PMA and PMA treated Warwick soil sampled on day 30 incubated at 5 ^{o}C and 25 ^{o}C. Error bars represent mean ± SD; n=3; *p<0.001. **(D)** Turnover of *D. nodosus* DNA in soil *rpoD* genome equivalents; soil inoculated with live, dead, UV treated, and free DNA (Error bars represent ± SD).

3.4.4. Turnover of *D. nodosus* DNA in soil.

DNA turnover in Warwick soil was fastest for free DNA (over 10 days) followed by UV and heat- treated cells. The live and intact UV treated cell survivals were not significantly different (Figure 3.5D and Table 3.7). Rates of decline were biphasic for all with change in rate as follows: day 4 for heat-treated; day 7 for free DNA; day 10 for live cells and day 14 for UV treated cells (Figure 3.4D).

Table 3.7. Rates of decay per day of *D. nodosus* DNA from various sources at 25 °C over two time periods.

Cell type	Decay rates (DNA decrease k day $^{-1}$)	
Live	D0-D10	D10-D60
	0.521	0.007
UV treated (Dead intact)	D0-D14	D14-D60
	0.415	0.007
Heat treated (Dead)	D0-D4	D4-D60
	1.502	0.013
Free DNA	D0-D7	D7-D60
	1.335	0.003

3.5. Discussion.

This is the first study to demonstrate that viable *D. nodosus* is detectable for 40 days off host in microcosms with aeration vents and that survival rate is affected by moisture, temperature and soil type. This is a step change in our understanding of survival of *D. nodosus* off host. A previous study of transmission of *D. nodosus* from floor to sheep indicated a 10-day off host survival of an infectious dose, defined by causing disease (Whittington, 1995) and a microcosm study reported detection of *D. nodosus* for 14 days (Cederlof et al., 2013). In the current study, populations of *D. nodosus* ~ 10^6 cells

were detected by qPCR in desiccated, cool, clay soil (Stockton) after 30 days (Figure 3.5). Given that approximately 10^2 cells were viable at day 30 in Warwick (non-clay) soil where qPCR was 10^4, it is likely that $>10^4$ cells were viable in clay soil at day 30 because of the overall higher load. In addition, the analysis of mRNA in the current study supported the observation of a significant viable *D. nodosus* population of 1.1 x 10^4 at 21 days; this was in microcosms with constant moisture content. Although it is not possible to determine whether 10^4 is an infectious dose, it is a similar load to that detected on healthy and footrot affected feet by (Witcomb et al., 2014) and highlights that certain soils, in cool damp conditions, might be a source of an infectious dose of *D. nodosus* for more than 30 days.

Non-sterile soil microcosms with four contrasting soil types were used that allowed soil to undergo drying to mimic the environmental conditions, survival in a range of MP's in soil can significantly impact on survival (Rattray et al., 1992). The wetting and drying curves of the four soils were determined so that the correct volume of water was added to standardize MP at -33 kPa average field moisture content for the UK. During trial desiccations studies the MP varied from -33 kPa to -10,000 kPA in heavy clay soil of Stockton and from -33 kPa to -36.5 kPa in Basilicata, a sandy loam (Figure 3.6). This reflects the suction required extracting water as these soils dry out (Artz et al., 2006). Previous work focused on *D. nodosus* survival in soil but did not consider changes in MP nor soil type and reported survival up to 14 days at 5 ^{o}C and 7 days at 15 ^{o}C respectively but water content was kept constant and MP was not determined in the soil used (Cederlof et al., 2013).

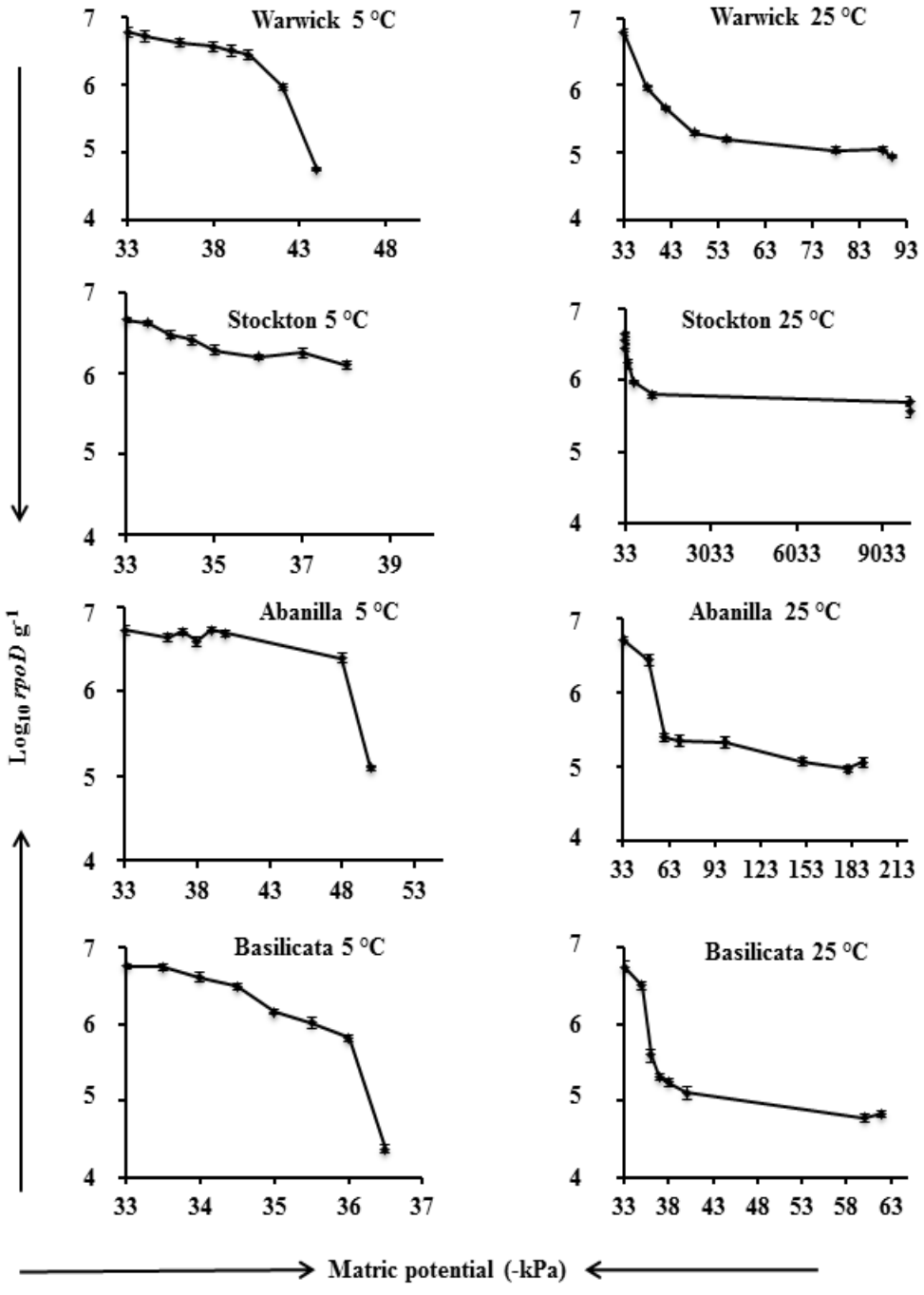


Figure 3.6. Decline in matric potential within soil types Warwick, Stockton, Abanilla and Basilicata over 40 days at 5 °C and 25 °C.

Studies on the survival of bacterial pathogens in soil have used both viable cell counts (Locatelli et al., 2013; Ma et al., 2014; Wang et al., 2014a) and molecular assays (Ibekwe et al., 2011). In the current study we used both because colony counting of *D. nodosus* is not possible because it does not form distinct colonies on the plate due to twitching motility: qPCR was used to enumerate cells and then RNA extraction and an in situ viability dye (PMA) were used provide a robust measure of viability. There was a significantly lower viable count than total count in the current study but still moderate loads of viable cells. Very few studies have used PMA on starved cells in soil therefore further work is needed to investigate cell integrity during survival.

The most significant reduction in *D. nodosus* DNA occurred in the first four days after inoculation with free DNA when water was present, in contrast, *D. nodosus* DNA persisted in soil for up to 60 days when the microcosm had dried out and prevented further degradation of DNA after the first week. Similar trends in turnover of free DNA and degradation were reported for *Mycobacterium bovis* DNA (Young et al., 2005). Their study also demonstrated that dead cells degraded quickly and that DNA did not persist at normal field moisture content for Warwick soil (Young et al., 2005). Thus it is likely that *D. nodosus* DNA detected in the current study is from living or, at worst, recently dead cells.

The biphasic decline in *D. nodosus* load in all soils related to water content indicates the critical drying out MP of each soil. In the case of Stockton this was -1033 kPa at 25 ^{o}C

but only -58 kPA in Abanilla. It is plausible that bacterial cells are so small that they do not respond in the same way to large changes in MP. Stockton soil showed the least decline in percentage water loss and survival mirrored changes in water content.

qPCR provided clear evidence of different t_d by soil type with slowest t_d in heavy clay soil (Stockton). Edaphic factors are critically influential as clay soils can hold more water than sandy soils and dry out more slowly, however, in this experiment the MP increased dramatically in clay soil as drying out resulted in greatly increased suction. This did not impact on *D*. *nodosus* survival, in contrast to the impact of MP on plants (Arias et al., 2000), and therefore strongly suggests that bacteria can survive within clay lattices as they dry out but retain water with *D*. *nodosus* surviving in a clay soil with MP of ~-10,000 kPa. It is likely that the clay content improved survival of culturable cells in Stockton despite the high suction required to access water in a dry clay soil. Gram-negative bacteria studies in soil have demonstrated that clay content (Locatelli et al., 2013); water availability (Rattray et al., 1992); organic carbon (Wang et al., 2014b) and strain type (Ma et al., 2014) could all markedly impact survival, with a general trend of heavy, wetter clay soils with higher organic carbon aiding survival. Other features of clay include buffering capacity and better nutrient availability (Bitton et al., 1972; England et al., 1993; Heynen et al., 1988). The decrease in particle size due to increased clay content increases the surface area of the soil and may result in protection from parasites, predators, toxins, UV and antibiotics (Höper et al., 1995; Johnson et al., 2006; KC, 1975). The type of clay also influences survival and more work is needed on this. Selected studies have implicated the role of the natural community as a key driver in the

survival of pathogens in soil where for certain soils the community had a negative effect on survival (Moynihan et al., 2015). Following our analysis of the physicochemical properties affecting *D*. *nodosus* survival, work is needed to address the microbial community impacts but these are likely to be soil specific.

3.6. Conclusions

From these findings, we conclude that for the Gram-negative pathogen *D*. *nodosus,* a temperature of 5 ^{o}C compared with 25 ^{o}C, and clay soil compared with other soil types, allowed survival of greater loads of *D*. *nodosus* over time than the previously reported studies. The results from field trials in Australia and from farmers' empirical experience in the UK suggest that sheep are not at risk from *D*. *nodosus* infection once fields have been rested for 14 days. The current study has indicated that *D*. *nodosus* can survive in soil with viable cells detected at day 40, and tolerate ultra-low soil MPs; further work is warranted to determine how clay impacts pathogen survival in soil and whether surviving cells are infectious, and at what dose, and work investigating presence of cells from field samples would provide information on infection load in rested fields.

CHAPTER 4

Genomic diversity and distribution of virulence determinants in UK isolates of *Dichelobacter nodosus*.

4.1. Abstract.

Footrot is the main cause of lameness in the UK and a major animal welfare issue. Multiple strains of the essential causal agent *D. nodosus* co-exist on the individual feet and show a markable level of diversity. To determine the relatedness of the UK *D. nodosus* isolates to a global population, a recent genome study has been completed between the global strains predominantly from Australian and Scandinavian and provided the evidence that the isolates on the basis of SNP analysis divided into two main clades, clade I and clade II. The clades I and II correspond to the virulent and benign respectively. The key genotype correlating with this bifurcation was the thermostable protease *aprV2* belonging to clade I, *aprB2* with clade II.

It was hypothesed that clade I contained predominantly virulent strains from the UK with clade II comprised of mainly benign strains. Further allele *pgr* is also an indicative gene with two homologs *pgrA* in virulent strains and *pgrB* in benign. The aim of the current study was to determine the relatedness of the UK isolates to a global *D. nodosus* population and also study expression of *pgr* gene under different condition particularly those conducive to the induction of virulence determinants. SNP analysis of UK isolates indicated that two main clonal populations existed that represented two clusters α and β within clade I. All the UK isolates were virulent based on the presence of *aprV2* and were recovered from clade I. This included a known benign strain BS4 from the UK containing an unstable protease and the genotype *aprB2*. Clusters α and β were a mix of *pgrA* and *pgrB* but both were in clade I. This contradicts with the role of *pgrB* as a non-virulent allele although expression of *pgrA* was induced by hoof horn in vitro whereas hoof horn showed no effect on the expression of *pgrB*.

4.2. Introduction.

Ovine footrot is a debilitating and highly infectious disease of sheep caused by an aerotolerant anaerobic bacterium *Dichelobacter nodosus* (*D. nodosus*) (Beveridge, 1941). The disease is characterized by inflammation of the interdigital skin with or without separation of the keratinous hoof from the underlying dermis. Both conditions result in lameness and loss of body condition (Egerton et al., 1969; Kennan et al., 2011; Stewart et al., 1984). Footrot is present worldwide; a main cause of lameness in the UK (Kaler and Green, 2008) and a major animal welfare issue (Fitzpatrick et al., 2006). A large number of factors contribute to footrot in sheep, the most important being the presence of the pathogen and climatic conditions combined with host susceptibility e.g. poor foot integrity (Kaler et al., 2010b). Increasing rainfall and temperatures favour the spread of footrot in sheep (Smith et al., 2014), however, many farmers report the highest prevalence of footrot in ewes in winter, possibly because ewes are housed or left untreated (Wassink et al., 2003).

Essential virulence factors of *D. nodosus* are its type IV fimbriae that allow *D. nodosus* to reach to the site of infection and thermostable proteases that cause the tissue degradation (Billington et al., 1996). These proteases form the basis to differentiate *D. nodosus* into benign and virulent strains and are widely used for diagnosis (Dhungyel et al., 2013; Rood et al., 1996) and further correlate with sequence differences between genes coding for extracellular proteases *aprV2* (virulent) and *aprB2* (benign) (Kennan, 2014). Recently a putative attachment factor Pgr was identified by Calvo-Bado (Calvo-Bado et al., 2011a) which suggested a role for this protein in mediating attachment to

the extracellular matrix within the hoof. *pgr* gene has two variants *pgrA* and *pgrB* and both variants have repeat regions that vary in the copy number between *D*. *nodosus* isolates. Bioinformatic analysis of Pgr indicated two transmembrane domains and two repeat regions R1 and R2 represent collagen alpha chain motifs and collagen-like cell surface motifs. It has also been postulated that Pgr protein may be partially or fully exposed at the surface of the cell and the transmembrane domains might be responsible for *D*. *nodosus* attachment to the foot (Calvo-Bado et al., 2011a).

The genome of *D*. *nodosus* was sequenced (Myers et al., 2007) and has a smallest genome of approximately 1.4 Mb. Considerable evidence for lateral gene transfer was indicated by codon usage including the identification of an integrated Mu-like bacteriophage. Virulence regions were included in these variable regions and transformation was most likely the process responsible for gene acquisition. Genomic islands were identified that included integrases and plasmid type genes which were associated with virulence islands. In addition 80 immunogenic proteins were identified by screening against ovine antisera and were recommended as candidate antigens for cross protective vaccine (Myers et al., 2007). Further genome analysis has been achieved by sequencing a number of diverse strains (n=103) from Australia, Bhutan, Nepal, India, Denmark, Norway, Sweden and UK (Kennan, 2014). All the *D*. *nodosus* isolates were highly conserved with > 95 % sequence identity supporting the mono-specific status of this genus. Comparative genome analysis of 103 sequences revealed *D*. *nodosus* strains divided into two distinct clades I and II based on the analysis of single nucleotide polymorphisms (SNP's) of 31,627 nucleotides which equates to 2.2 %

of the genome. The two clades appear to correlate with benign and virulent phenotypes based on previous studies. Analysis of the strain data indicated that 98% in clade I carried *aprV2* protease gene and were regarded as the virulent group whereas the majority (97%) of clade II carried the *aprB2* benign ortholog. The division into clade I and clade II did not take the geographical origin of the isolates into account but most of the strains (88 %) were from Australia and Scandinavia. Only one strain was representative of United Kingdom and this was a known virulent strain.

The current study was an opportunity to include genomes from UK virulent and benign isolates of *D*. *nodosus* and establish their relationship to the defined clades I and II. Further information on the distribution of virulence regions within the clades would also be useful but was lacking in the genome study due to the high variability of regions such as VAP 1, VAP 2, VRL and PGR. The latter region is one of the considerable interest in the current study and therefore the distribution of the two homologs A and B within the clades will be of interest to determine if a correlation exists with pathogenicity. We hypothesize that *pgrA* will be correlated with pathogenicity and that UK isolates will distribute according to their *aprV2*/*B2* and *pgr* genotypes. The following research was aimed at selecting a representative range of UK isolates from a recent cohort study for comparison with the recent genome study of 103 isolates in order to establish the relationships between virulence, geographic isolation and pathogenicity factors. In addition, two *D*. *nodosus* strains representing A and B homologs of *pgr* were used for studying transcriptional profiles of *pgr* expression under different conditions to determine the role of hoof horn for gene induction and correlation with virulence as

defined by phenotype protease gelatin gel assay and possession of the *aprV2* homolog.

4.3. Materials & Methods

4.3.1. Herd details, sampling and Isolation of *D. nodosus*.

A cohort study was initiated at Bristol University Vet School using a flock of 100 mule and Suffolk cross bred sheep from which 50 were chosen in October 2010 based on the history of lameness with an aim to study the diversity of *D. nodosus* strains and impact of various footrot treatments on the severity of disease. The ewes were mated to five Texel and Suffolk crossbred rams in October 2010. Selection of the final 50 sheep was achieved as follows; the ewes were divided into two groups of 50 each based on the footrot and interdigital lesion scores and body condition, each ewe was given an ID starting from 0051 – 0150. From these two groups, a cohort of 25 ewes from each group were selected by stratified random sampling using interdigital dermatitis and footrot scores according to (Kaler and Green, 2009b) and body condition at the time of examination, thus creating a focus cohort of 50 ewes that were studied in detail at four non-consecutive four-week periods i.e. October/November 2010, January/February 2011, March/April 2011 and August 2011. Lambs were born in March/April 2011 and integrated in the study (Appendix 6).

At each time point, swab samples were taken from the interdigital space from the ewes and lambs when they were born for isolation of *D. nodosus* using sterile cotton swabs (EUROTUBO collection swab; Delta lab, Rubi, Spain). Another swab sample was taken from the same foot to complete molecular analysis. Swabs were streaked onto 4 %

trypticase arginine serine hoof (TASH) agar plates (Table 4.1) in the field and the plates were packed in Anaerobic Jars containing Anaero gas packs (AnaeroGen, Oxoid, Ltd, UK) and incubated at 37 ^{o}C for four days. The colonies observed on the agar were subcultured into trypticase arginine serine (TAS) broth and incubated anaerobically at 37 ^{o}C for four days. Growth was harvested and used for DNA extraction with NucleoSpin Tissue Kit (Macherey-Nagel, GmbH and Co, Duren, Germany).

As mentioned above, duplicate swabs were collected from all four feet of sheep held at University of Bristol Vet School for the purpose of isolation of *D. nodosus* and DNA extraction. Each month, four visits were made to the Bristol Vet School (one visit per week) for sample collection. The swabs for isolation of *D. nodosus* were collected only at the first week out of four week period but the swabs for molecular analysis were taken at all four weeks every month during 10 month sampling period. The swabs for *D. nodosus* isolation were immediately streaked on to 4% TASH agar plates after collection which were then placed in an Anaerobic jar containing Anaero gas and incubated at 37 ^{o}C for four days at a laboratory in University of Bristol Vet School. After four days, the pure colonies/isolates observed on 4% TASH agar plates were lyophilized for long term storage and data on the number of isolates per foot were recorded. The swabs for molecular analysis were stored at 4 ^{o}C for transportation to the laboratory at Warwick University and stored at – 80 ^{o}C until further processing.

Table 4.1: Composition of TASH agar (1 litre)

Reagents	Quantity in grams
Protease peptone	5.0
Lab Lemco	5.0
Yeast extract	2.0
Peptone from Casein	15.0
L-Arginine	5.0
DL-Serine	1.5
L-Cysteine	0.405
$MgSO_4$	2.0
Agar	20.0
HEPES	25.0
$CaCl_2$	20.0
Hoof Powder	15

4.3.2. Extraction of bacterial DNA from swabs.

Total genomic DNA was extracted from the swabs using the NucleoSpin Tissue Kit (Macherey-Nagel, GmbH and Co, Duren, Germany) with some modifications. Swabs were thawed at 4 ^{o}C and 400 ml of buffer T1 was added followed by 40 ml of proteinase K. The samples were vortexed twice for 5 s and incubated for 10 min at 56 ^{o}C. The mixtures were transferred to microcentrifuge tubes and 400 ml of buffer B3 was added. The samples were vortexed twice for 5 s and incubated for 5 min at 70 8C then allowed to cool before adding 400 ml of 100% ethanol. The samples were again vortexed twice and the supernatant transferred to a NucleoSpin Tissue column and centrifuged at 11,000 x g for 1 min. The flow-through was discarded, the membrane was washed with 500 ml of buffer B5 and centrifuged at 11,000 x g for 1 min. The flow-through was again discarded; the column was washed with 600 ml of buffer B5 and centrifuged at 11,000 x g for 1 min. The flow-through was again discarded and the membrane dried by

centrifugation at 11, 000 x g for 1 min to remove residual ethanol. The DNA was eluted into 40 ml of elution buffer, warmed to 70 ^{o}C and centrifuged at 11,000 x g for 1 min and the resultant DNA was stored at -20 ^{o}C for further use.

4.3.3. Characterization of isolates and selection for whole genome sequencing.

All the isolates were characterized for serogrouping (Claxton et al., 1983), Multi Locus Variable Number Tandem Repeat Analysis (MLVA) based on the number of repeats at DNTR02, DNTR09, DNTR10 and DNTR19 loci (Russell et al., 2014) and *pgrA*/*B* homolog using different primers pairs (Calvo-Bado et al., 2011a). *D*. *nodosus* strains (n=22) were chosen for genome analysis on the basis of phenotype and genotype to represent the population diversity (Table 4.2).

Table 4.2. Phenotypic and genotypic characteristics of the isolates used in the study.

Barcode	Sheep ID	Month	Foot	Serogroup	*pgr*	MLVA Type	DNTR02	DNTR09	DNTR10	DNTR19	Ewe/Lamb	ID lesion score	FR lesion score
03561560	0147	Nov	LF	B	A	114	15	6	11	3	Ewe	3	0
03561904	0147	Nov	LF	B	A	114	15	6	11	3	Ewe	3	0
03562598	0147	Feb	LF	B	B	67	7	7	12	5	Ewe	0	0
03565117	0147	Aug	LF	B	A	129	19	5	6	4	Ewe	2	0
03562239	0111	Jan	RR	B	A	114	15	6	11	3	Ewe	0	0
03564158	0111	Aug	RR	B	A	124	18	5	6	3	Ewe	1	0
03563250	0121	Apr	LR	B	B	66	7	7	12	4	Ewe	4	0
03563366	0121	Apr	LR	B	B	65	7	7	12	3	Ewe	4	0
03564783	0121	Aug	LR	B	B	66	7	7	12	4	Ewe	1	0
03565025	0121	Aug	LR	B	B	66	7	7	12	4	Ewe	1	0
03562475	0088	Jan	RR	B	A	52	5	6	11	3	Ewe	1	0
03563953	0272	May	LR	I	B	60	6	7	12	4	Lamb	0	2
03563588	0321	May	RF	B	B	69	8	7	12	4	Lamb	4	0
03561584	0112	Nov	LF	B	A	81	11	6	10	3	Ewe	4	0
03562451	0088	Jan	LR	B	A	81	11	6	10	3	Ewe	1	0
03561508	0149	Nov	RF	B	B	87	11	7	11	4	Ewe	0	1
03562444	0088	Jan	LR	B	A	90	12	6	10	3	Ewe	1	0
03562062	0124	Nov	RR	B	A	102	13	6	11	3	Ewe	1	0
03561577	0114	Dec	LF	H	A	105	14	5	6	5	Ewe	0	1
03561676	0147	Nov	LF	B	A	108	14	6	11	3	Ewe	3	0
03564387	0059	Aug	RR	I	A	142	36	6	12	3	Ewe	4	0
03562055	0147	Nov	RR	I	A	145	40	6	11	3	Ewe	4	0
BS4	*intA* and *vap* - +ive; *vrl*, gelatin gel -ive; Serogroup C, *pgrB*.												
VCS1703A	*intA*, *vap*, *vrl*, gelatin gel +ive; Serogroup G *and pgrA*.												

4.3.4. Genome Sequencing of Isolates.

The lyophilised stocks previously made were re-suspended in 200 µl TAS broth and the suspension was plated on to the 4 % hoof agar plates which were incubated in the anaerobic cabinet (Don Whitney Scientific, UK) at 37^{o}C for 4-5 days. Where good growth was obtained DNA was extracted using DNeasy Blood and Tissue kit (Qiagen UK). In addition previously extracted DNA from these isolates was used if poor growth was recovered. The purity of the DNA from each sample was confirmed by a *D. nodosus* 16S rRNA specific end point PCR using the primers Cc and Ac (Calvo-Bado et al., 2011a). The PCR products were purified using the PCR purification Kit (Qiagen, UK) by following the manufacturer's instructions and sequenced using GATC biotech (London, UK) using primers Ac and Cc. All the sequences obtained were trimmed and blasted against the NCBI Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences that showed 100 % identity to *D. nodosus* were included for next generation sequencing (NGS).

The whole genome shotgun method was used to produce a draft genome sequence for each of the *D. nodosus* isolates. DNA was quantified fluorometrically using Qubit (Life Technologies). 1 ng of DNA was used for sequencing library construction, using NexteraXT, following the manufacturers` instructions. Sequencing was carried out on a Illumina MiSeq using V2 chemistry to produce 2 x 250 bp reads.

The read datasets for each of the isolates filtered to remove low quality bases and sequencing adapters (Tool used: Trimmomatic). Reads that were left with less than 30

bases were discarded. Filtering removed less than 4% of the reads. The filtered reads were assembled using Velvet, the optimal K-mer value was estimated using KmerGenie. Prior to annotation, contigs were sorted using the VCS1703A closed genome sequence as a guide (Assume genome synteny) using Mauve. The draft genome sequence for each isolate was annotated using Prokka. The relationship between the core genomes of each of the isolates was inferred using both Wombac and Nesoni NWAY.

4.3.5. *pgr* sequencing and phylogenetic analysis.

As mentioned above, *pgr* is a polymorphic protein with two variants *A* and *B*, both have repeats regions that vary in number of tandem repeats; the size of the whole *pgr* gene is approximately 3.3 Kb. Attempts were made to amplify the whole gene from the UK isolates using *pgrF* and *pgrR* primers (Calvo-Bado et al., 2011a). However, it was difficult due to the increasing number of repeats except for the few isolates where a very faint band was observed and these were not processed further. The sequence of *pgr* gene was divided into three regions 1, 2 and 3 and new primers were designed from the reference genome sequence of the type strain VCS1703A (*pgrA*) (Table 4.3) to target regions 1-3. These primers were tested on a collection of strains held at University of Monash which included strains from various geographical locations. However, when these primers were tested on the UK strains, no amplification was observed in some of the isolates after many attempts in region 2 that incorporates the R2 tandem repeats.

Table 4.3: Newly designed primers for amplification of *pgr* gene regions.

Primers targeting different regions of the *pgr* gene (designed from VCS1703A sequence).	**Sequence (5'- 3')**
Region 1 forward	GCATTGGCAAGCGCAAA
Region 1 reverse	TGACGGTAGCAGTTGGA
Region 2 forward	GTGGTGAGAACACTGAATTA
Region 2 reverse	CTATTACGATCAGTATTCAAG
Region 3 forward	AGCTTTTTCAGATGGTTCCGTAA
Region 3 reverse	GACATGGGCAACGTTGGCTCCAT

An alternate approach was chosen to amplify the variable region that was common to both *pgrA* and *pgrB* from the UK and Australian strains. DNA used in the genome study for strains from Australia, Denmark, Nepal and India together with DNA from the 22 UK strains was amplified using *pgrF1* and *pgrR1* variable region primers (Calvo-Bado et al., 2011a). The PCR products were purified using PCR purification kit (Qiagen, UK) by following the manufacturer's instructions and sequenced by GATC biotech (London, UK) using *pgrF1* and *pgrR1* primers. All the sequences were trimmed and aligned to the reference sequence from the strain VCS1703A using MEGA6.06 (Tamura et al., 2013). The nucleotide sequences were translated to the amino acid sequences and neighbour joining trees were constructed using MEGA6.06 (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei, 2000) and are in the units of the number of amino acid differences per site.

4.3.6. Comparison of *pgrA* and *pgrB* upstream regions.

To determine if there were any differences in the promoters, the sequences immediately upstream of *pgrA* and *pgrB* of the 22 strains from the UK and a selected range of

Australian strains based on their *pgr* status (20 each of *pgrA* and *pgrB* strains) were aligned using Mega6.06 (Tamura et al., 2013). The promoters were predicted using the promoter recognition program BPROM (Solovyev, 2011). The layout of the *pgr* gene and the genes upstream and downstream in the strain VCS1703A are shown in (Figures 4.1, 4.2 and 4. 3).

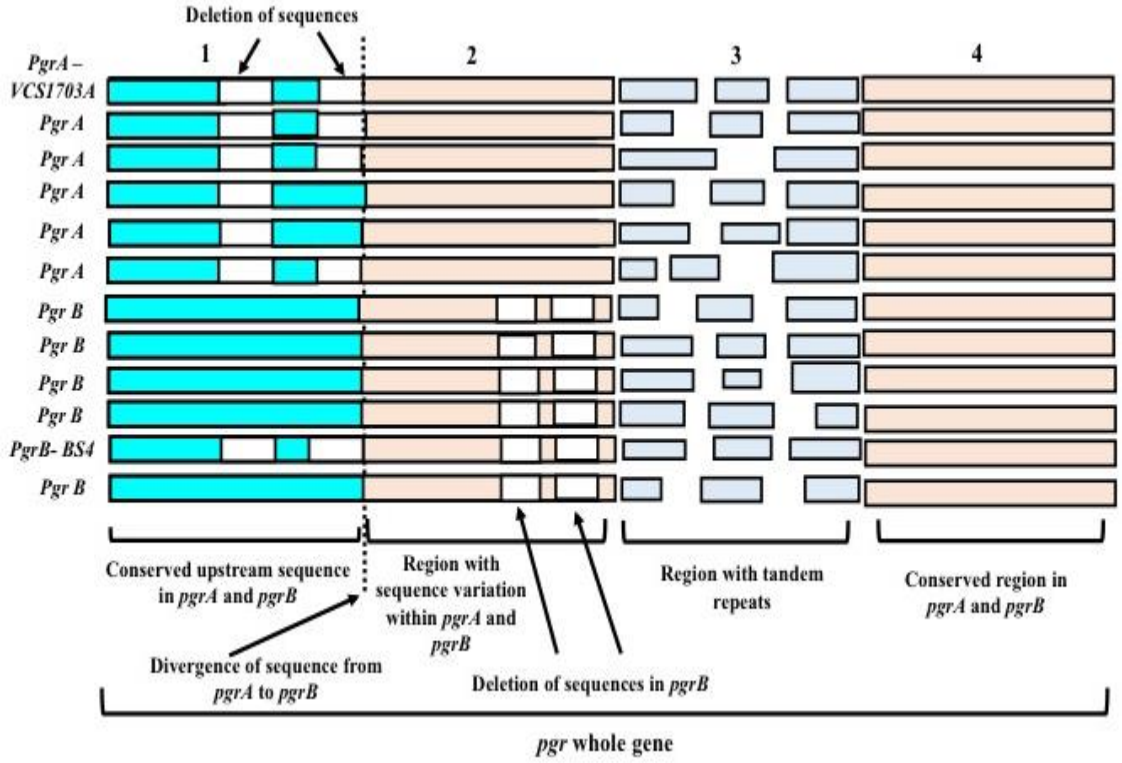


Figure 4.1: Layout of the *pgr* whole gene. The gene is divided into four regions 1, 2, 3 and 4. Region 1 - the upstream region conserved in all *pgrA* and *pgrB*. The region has a deletion of sequences present in all *pgrA* strains and some *pgrB* strains including BS4; Region 2 – Divergence of sequence from *pgrA* to *pgrB*, this region has high variation in the sequence between *pgrA* and *pgrB* and also contains a deletion of sequence in *pgrB*; Region 3 – region with tandem repeats in *pgrA* and *pgrB*; Region 4 – another conserved region downstream to the region with tandem repeats in *pgrA* and *pgrB*.

Figure 4.2: Graphical overview of the *pgr* gene region of the VCS1703A strain of *D. nodosus*. The view is a screen shot from Artemis (Sanger.ac.uk) showing protein coding regions as blue boxes. The *pgr* gene is labelled as DNO_0690 and is encoded on the reverse strand between bases 745275 and 741913 (3363 bases, 1120 amino acids). The gene is encoded on the reverse strand and is located between genes annotated as *mfd* and *era*, both encoded on the reverse strand.

rnhB	734321	734890		ribonuclease HII
dnaE	734925	738452		DNA polymerase III, alpha subunit
mfd	738458	741811	c	transcription-repair coupling factor
DNO_0690	741913	745275	c	hypothetical protein
era	745734	746633	c	GTP-binding protein Era
rnc	746630	747331	c	ribonuclease III
lepB	747331	748302	c	signal peptidase I
lepA	748315	750117	c	GTP-binding protein LepA
DNO_0695	750343	751140		conserved hypothetical protein
DNO_0696	751142	751600		conserved hypothetical membrane protein
DNO_0697	751611	752738		peptidase M20 family
DNO_0698	752830	754590		sulfate transporter family protein

Figure 4.3: CDS genes, their function and location on the chromosome (VCS1703A). *pgr* is located upstream to GTP binding protein *era* and downstream to transcription-repair coupling factor *mfd*. The view is a screen shot from Artemis (Sanger.ac.uk).

4.3.7. Prediction of *PgrA* structure.

The amino acid sequence of the wild type reference strain VCS1703A (*pgrA*) having the reference number NC_009446.1, was obtained from National Centre for Biotechnology Information (NCBI) reference sequence database. The three dimensional structure of the amino acid sequence was determined using the Phyre2 web portal for modelling of the protein, prediction and analysis (Kelley et al., 2015). The dimensions of the model were (A): **X**:51.439; **Y**:69.297; **Z**:58.955.

4.3.8. Determination of the expression of the Pgr locus of *D*. *nodosus*.

Virulent and benign strains of *D*. *nodosus*, VCS1703A (type species) and BS4 (benign UK strain) were cultured on TAS agar (Table 4.1), except the hoof horn powder was not added) in an anaerobic cabinet and incubated for 4-5 days at 37 °C. After five days of incubation, the colonies were Gram stained and examined for purity and identification of *D*. *nodosus*. The cells from both strains were harvested separately in 2 ml phosphate buffer saline and concentration of the harvest was determined by hemocytometer at 10^8 CFU/ml. The cells from both strains were then sub cultured separately onto twenty four 2% hoof agar (TASH) plates (twelve plates each for DNA and RNA extraction over four time points) and twenty four non-hoof (TAS) agar plates so as to obtain three biological replicates at each time point. The plates were incubated anaerobically and harvesting of the cells from hoof and non-hoof agar plates was carried out at days 0, 3, 6 and 8.

4.3.9. Extraction of bacterial DNA from cultured cells.

DNA was extracted from the *D*. *nodosus* cells using the DNeasy Blood and Tissue Kit

(QIAGEN, UK) according to the manufacturer's recommendations with some modifications. The concentration of approximately 10^7 cells was centrifuged for 5 min at 5000 x *g* in 2ml eppendorf tubes (Eppendorf, Hamburg Germany). The supernatant was removed and pellet resuspended in 400 µl of phosphate buffer saline (PBS) to which 40 µl of proteinase K was added. To this mixture, 400 µl lysis Buffer AL was added. The suspension was thoroughly mixed by votexing and incubated at 56 °C for 10 min. Subsequent to this, 400 µl of 100 % ethanol was added to the tubes followed by mixing by votexing. The mixture was pipetted into DNeasy Mini spin column placed in a 2 ml collection tube supplied with the kit. The column was centrifuged at 8000 x *g* for 1 min and flow discarded along with the collection tube and the spin colum was placed in a new collection tube. The column was washed with 500 µl of Buffer AW1 and centrifuged at 8000 x *g* for 1 min. The flow was discarded along with the collection tube. The spin column was placed in a new collection tube and 500 µl Buffer AW2 was added and column centrifuged at 20,000 x *g* for 3 min. The flow was discarded along with the collection tube. To elute the DNA, the spin column was transferred into a sterile 1.5 ml eppendorf tube and 70 µl of Buffer AE was added onto the spin column membrane. The column was incubated for 1 min at room temperature and centrifuged at 8000 x *g* for 1 min. The resultant genomic DNA from the strains VCS1703A and BS4 were used as a standard to estimate the amount of *pgrA* and *pgrB* from the cDNA.

4.3.10. Extraction of bacterial RNA from cultured cells.

RNA was extracted from *D. nodosus* cells grown on TAS agar with and without hoof horn and agar was used to avoid extraction of suspended solids from the hood horn in a

liquid medium. The RNeasy Mini Kit (QIAGEN, UK) was used for extraction of RNA according to the manufacturer's instructions with some modifications. All the steps were carried out on ice. The cell concentration of approximately 10^7 cells determined by haemocytometer was placed in 2 ml nuclease free Eppendorf tubes and centrifuged at 800 x *g* for 10 min. The supernatant was removed and 700 µl of Buffer RLT and 700 µl of 70% ethanol was added to the cells. The suspension was thoroughly mixed and approximately 700 µl of this suspension was added to the RNeasy mini spin column placed in a 2 ml collection tubes that were supplied with the kit and centrifuged at 8000 x *g* for 15 s. The remaining sample was processed in the same manner until completed. To wash the RNA bound to the spin column membrane, 700 µl of Buffer RW1 was added to the column and centrifuged at 8000 x *g* for 15 s. The flow-through was discarded along with the collection tube and the spin column was placed in a new collection tube. Second washing was carried out by adding 500 µl of Buffer RPE to the spin column and centrifuged at 8000 x *g* for 15 s then for a further 2 min. The eluate was discarded and spin column was placed in a new collection tube. Finally, RNA was eluted by adding 40 µl of RNase free water onto the spin column membrane and centrifuged at 8000 x g for 1 min. The resultant RNA was stored at -20 °C.

DNase treatment of the RNA samples was carried out using Turbo DNase free Kit (Applied Biosystems, Warington, UK) following the manufacturers recommendations. cDNA was synthesised from DNA-free RNA using High capacity RNA-to-cDNA Kit (Applied Biosystems, Warrington, UK) by following the manufacturer's recommendations. The resultant cDNA was stored at -20 C for further use.

4.3.11. Normalization using *rpoD*, *D. nodosus 16S rRNA*, *pgrA* and *pgrB* from cDNA.

Expression levels of the target genes (*pgrA* and *pgrB*) and reference genes (*rpoD* and *D. nodosus 16S rRNA)* were measured in cDNA samples, standards and no template controls (nuclease-free water) at 0, 3, 6 and 8 days, using the Applied Biosystems 7500 Fast real-time PCR system. All qPCR reactions were set up as described above with *rpoDF* and *rpoDR*; *D. nodosus 16SF* and *D. nodosus 16SR* (Calvo-Bado et al., 2011a); *pgrAF* and *pgrAR*; and *pgrBF* and *pgrBR* (Muzafar et al., 2015). The results were analysed using 7500 Fast System SDS software (Applied Biosystems). The fold change in the expression of target genes *pgrA* and *pgrB* was determined for days 6 and 8, relative to the levels at day 3 both with and without hoof horn. Housekeeping genes *rpoD* and *D. nodosus 16S rRNA* were evaluated for use as reference genes for normalization. Fold change in the expression of both housekeepers was calculated. The average gene copy number of the target genes *pgrA* and *pgrB* were divided by the average copy number of the reference gene *rpoD* to calculate the fold-difference in *pgrA* and *pgrB* expression relative to the *rpoD*. Since *rpoD* is present as a single copy number in *D. nodosus* chromosome, therefore selected as a reference gene for normalization. The normalised expression levels of *pgrA* and *pgrB* at day 3 were set as calibrator and assigned the constant value 1 and the fold change at days 6 and 8 were calculated relative to day 3. The normalization and calculation of fold differences was done as per (Bugrysheva et al., 2011).

$$\textbf{Normalised expression} = \frac{\textbf{Expression of the target gene}}{\textbf{Expression of the reference gene}}$$

4.4. Results.

4.4.1. Recovery of isolates.

The number of colonies/isolates per individual foot obtained on 4% TASH agar plates were recorded for estimation of total number of isolates obtained in the cohort study which is described in section 4.3.1. The occurrence of *D. nodosus* colonies on the TASH agar plates was observed at very low levels at approximately 4.13 % (Gilbert, 2013). There are many possible explanations for poor recovery as we anticipated *D. nodosus* to be ubiquitous by cultivation independent approaches (Muzafar et al., 2015). Reason for poor recovery may relate to competition with other bacteria, poor survival on agar and exposure to harmful conditions during swabbing and extraction.

A recent study on the analysis of molecular and evolutionary characteristics of *D. nodosus* by (Gilbert, 2013) on the same cohort described above reported the prevalence of a particular isolate (BB7-7-12-4, the numbers indicate repeats at each DNTR locus and designated as MLVA type 66) at 23.3 % with a total of 71 isolates obtained in the whole study (Gilbert, 2013). Whilst selecting the isolates for whole genome sequencing, BB7 (MLVA type 66) isolated from one foot in ewe 0121 at various time points (longitudinal sampling of the same animal, same foot over three different time points) was included in the genome sequencing to determine if there occurred any variations at genomic level in BB7 isolates obtained at three different time points from the same foot in the same animal.

All the isolates for genome sequencing were chosen from a total of eleven ewes and two lambs. First eight isolates were taken from a particular foot in three selected ewes 0147, 0111 and 0121 for which the isolates were obtained at a minimum of three time points over one year study period. The remaining 14 isolates were taken from seven ewes and two lambs. Most of the isolates taken from selected ewes and lambs had different MLVA profiles except the three isolates from a particular ewe with a single MLVA type 66 as observed for BB7. The interdigital dermatitis and footrot scoring for each isolate were also taken into account whilst making the selection. Overall, the selection of the isolates was based on a diverse range of parameters including serogroup, *pgrA*/*B*, MLVA type, footrot and interdigital dermatitis scoring of the individual feet from which the isolate was taken (Table 4.2).

4.4.2. Existence of Genomic diversity within *D. nodosus* isolates from the UK.

The isolates were selected for genome sequencing based on the genotypic and phenotypic information available on UK CEDFAS footrot database maintained at the laboratory of Professor's. Wellington and Green, University of Warwick. Each isolate represented by a unique identification number correspond to a unique set of phenotypic and genotypic characteristics for that particular isolate. This information is available on CEDFAS database and is presented in Table 4.4 to represent the set of isolates used in the current study. Using core genome comparison, the Bristol farm isolates were divided into two distinct clusters α and β in the clade I (Figure 4.4). Using the Wombac SNP calling protocol, no more than 160 core SNPs were observed in a pairwise comparison of isolates in each of the two clusters (Figure 4.4, Table 4.4). Between clusters, more

than 5000 SNPs were counted in each of the pairwise comparisons. Examining the distribution of SNP differences that are responsible for the differences between the clusters, an even distribution of these SNPs across the reference genome was observed. These differences are listed in Table 4 Core genome comparison.

The cluster α as indicated in red in figure 4.4 comprised of the isolates 03564783, 03563588, 03562598, 03561508, 03565117, 03565025 and 03563953 whereas Cluster β contained 03563250, 03562238, 03561676, 03562444, 03561904, 03562055, 03561584, 03562451, 03563366, 03562062 and 03564387. Also, in clade I, four singletons γ - 03562475, δ - 03571577 and ε - BS4 and VCS1703A were observed. All the UK isolates in clade I, contained the virulent protease *aprV2* except for the strain BS4, which is a known benign strain from the UK as indicated by having an unstable protease which was determined by the gelatin gel thermostability test. The grouping of the UK isolates into two main clusters α and β was irrespective of the *pgr* status of the given isolate (Table 4.2).

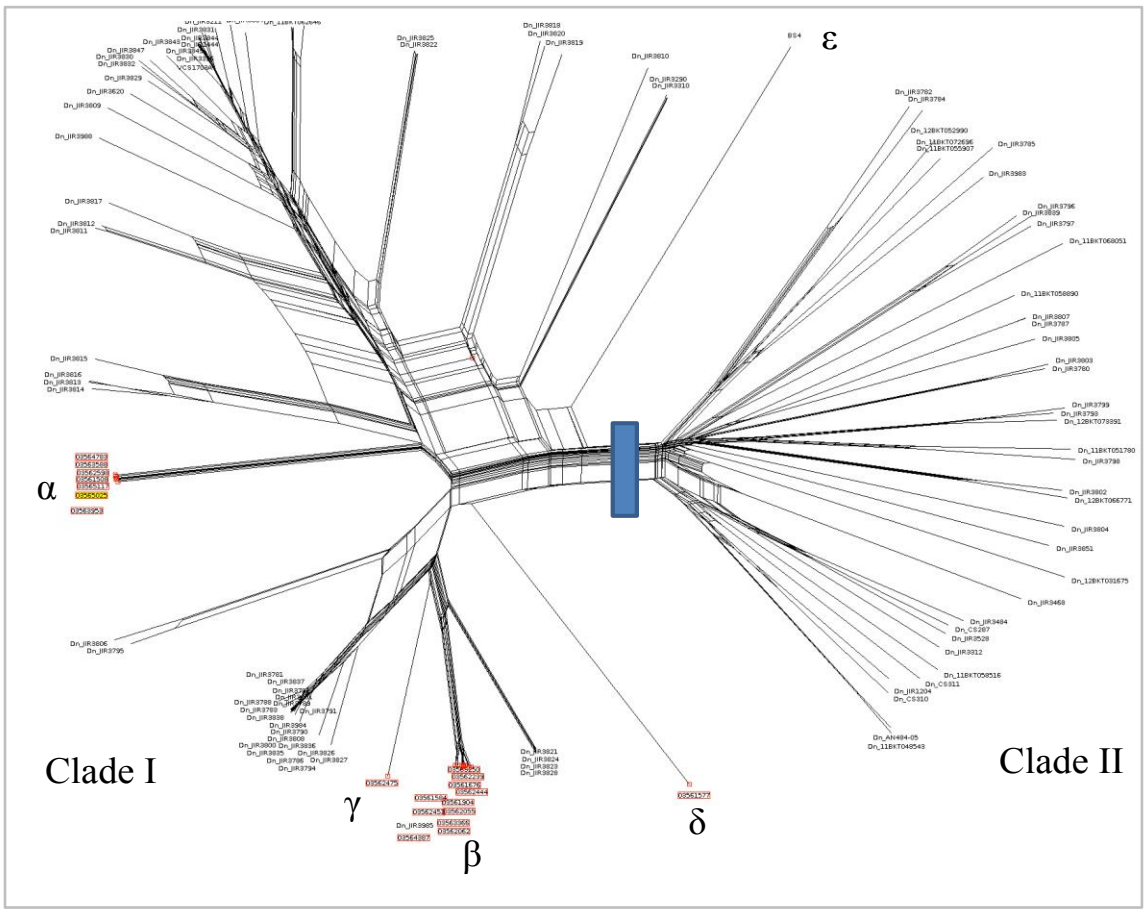


Figure 4.4: Tree showing the relationship between 102 *D. nodosus* isolates (Kennan et al, 2014). Clade 1 to the left and Clade II to the right. The tree shows the isolates from the previous study by Kennan et al., 2014 which are highlighted in black within clades I and II. The phenotypic and genotypic characteristics for each isolate represented in black can be obtained in the supplementary materials (Kennan et al., 2014). The tree also contains isolates from the UK used in the current study as indicated in red in clade I except strain BS4 present in Clade 1 and indicated in black. The isolates from the current study are divided into two main clusters α and β with three additional singletons γ, δ and ε. Cluster α contains the isolates 03564783, 03563588, 03562598 and 03561508, 03565117, 03565025 and 03563953. Cluster β contains the isolates 03563250, 03562238, 03561676, 03562444, 03561904, 03562055, 03561584, 03562451, 03563366, 03562062, 03564387. The singletons are γ - 03562475, δ -03571577 and ε - BS4. The numbers against each isolate presented here are the unique identification numbers which correspond to different phenotypic and genotypic characteristics. The data for each isolate has been stored in the UK CEDFAS footrot database held in the laboratory of Professor's Wellington and Green, School of Life Sciences, University of Warwick.

Table 4.4: Pairwise SNP differences between isolates from this study.

ID	3561904	3562239	3562598	3563250	3563366	3564783	3565025	3565117	Reference
3561904	0	146	5404	157	157	5405	5405	5383	6396
3562239	146	0	5398	127	127	5399	5399	5377	6396
3562598	5404	5398	0	5390	5390	53	53	43	6173
3563250	157	127	5390	0	0	5391	5391	5369	6416
3563366	157	127	5390	0	0	5391	5391	5369	6416
3564783	5405	5399	53	5391	5391	0	0	96	6174
3565025	5405	5399	53	5391	5391	0	0	96	6174
3565117	5383	5377	43	5369	5369	96	96	0	6190
Reference	6396	6396	6173	6416	6416	6174	6174	6190	0

4.4.3. Analysis of *pgr* variable region in *D. nodosus* strains.

Analysis of 37 amino acid sequences immediately upstream of *pgr* variable region divided the isolates into two main clusters *pgrA* and *pgrB*. The isolates from Australia, Denmark and India clustered with the UK isolates. All *pgrA* sequences clustered separately irrespective of the geographical location of the isolate. The UK *pgrB* isolates from the previous study and the current study clustered together (Figure 4.5).

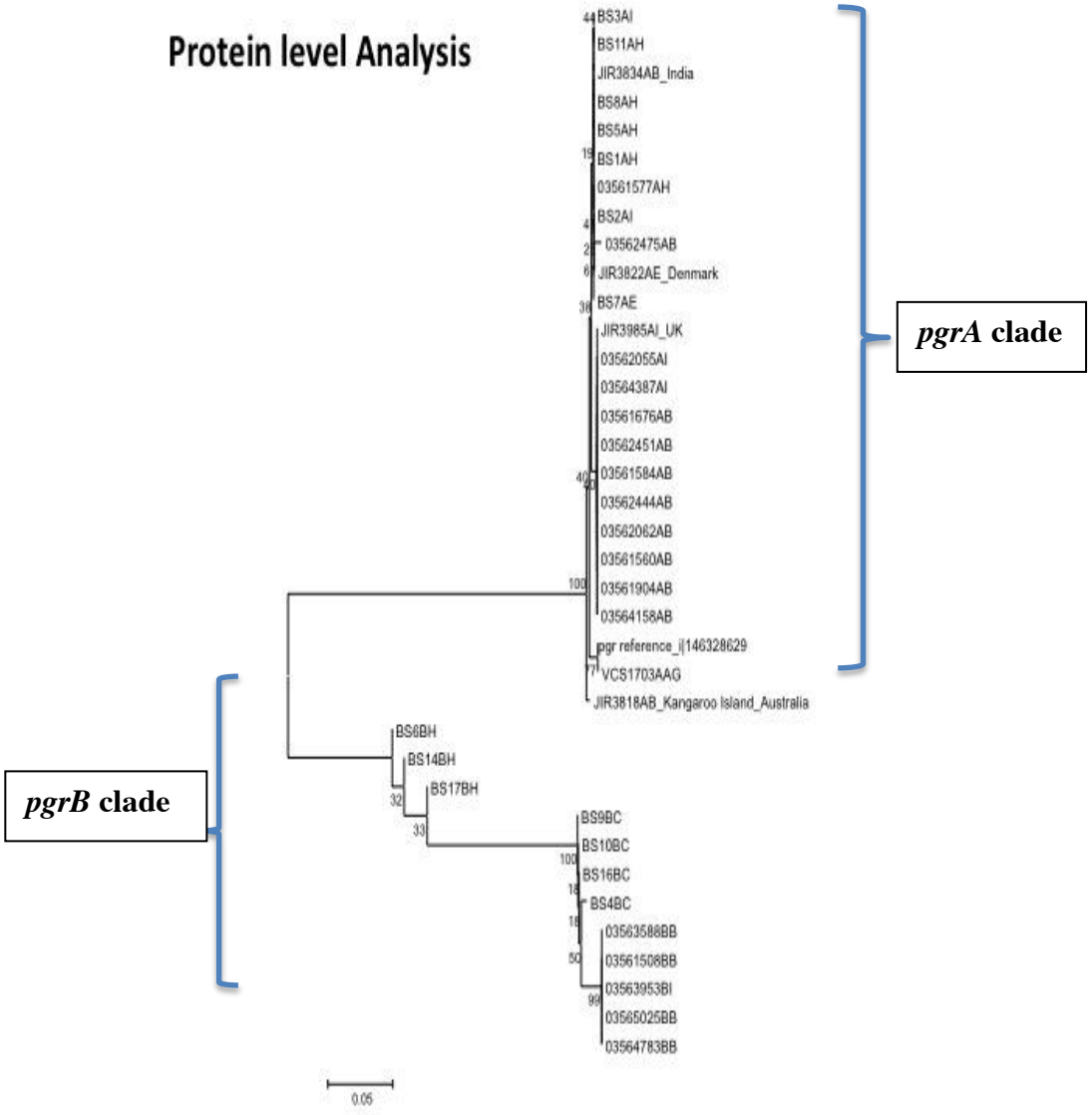


Figure 4.5: Evolutionary relationship of taxa based on the sequences from the *pgr* variable region. The strains with initials 'BS' are the UK strains collected from the Bristol University Veterinary School. The sequences were taken from a published study for comparison (Calvo-Bado et al., 2011a). The strains with initials 'JIR' were taken from the collection of 103 strains at Monash University, Australia. The strain ID's starting with '0356' were taken from the CEDFAS Bristol cohort for the current study.

4.4.4. Comparison of *pgrA and pgrB*.

Analysis of the sequences immediately upstream of *pgrA and pgrB* isolates indicated that both *pgrA* and *pgrB* isolates from the UK and Australia contained a conserved sequence of approximately 400 bp in the region 1. Furthermore, in *pgrA* isolates, region 1 contained 4 bp deletions of sequences at the site 545 on the sequence alignment and some isolates but not all contained another 4 bp sequence deletions at the site 570. In contrast, all *pgrB* isolates except BS4 and JIR3812 did not contain the deletion of sequences at both sites (Figure 4.6).

To determine if there was any variation in the promoters in strains VCS1703A (*pgrA*) and BS4 (*pgrB*), the sequences upstream to the start codon (ATG) near the deletion region 1 indicated that *pgrA* isolates had a different promoter compared to *pgrB*. However, in the strain BS4 (*pgrB*), the promoter sequence was identical to the promoter found in *pgrA* isolates (Table 4.5). This is because BS4 strain has an identical upstream region as *pgrA* (Figure 4.6).

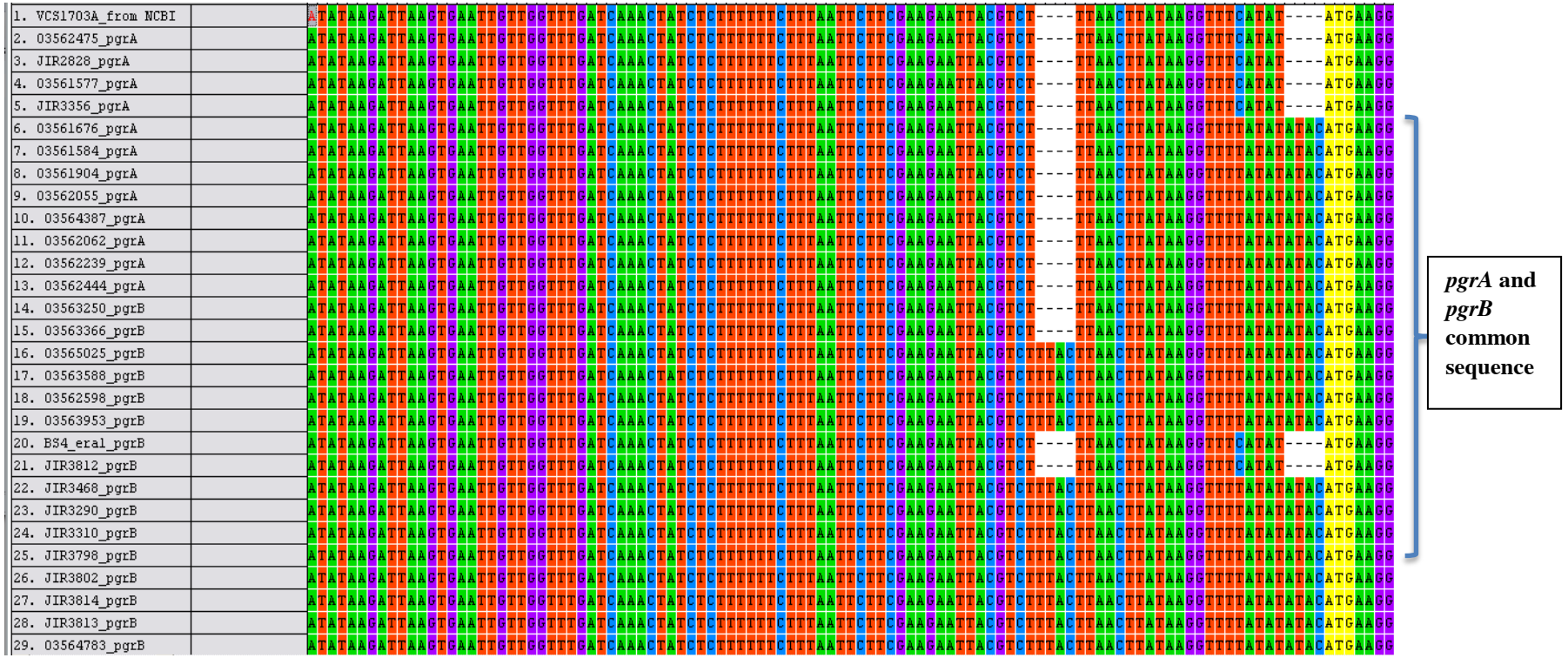


Figure 4.6: Conserved sequence and deletion of sequence in the region 1 in *pgrA* and *pgrB* isolates from the UK and Australia. The sequence highlighted in yellow is the start codon ATG.

Table 4.5: Predicted promoters in the strains VCS1703A and BS4.

Characteristics	VCS1703A and BS4
-10 box (sequence)	TTTTAGAAA
-35 box (sequence)	TTTACA
-10 box at position	128
-35 box at position	108
Number of predicted promoters	1
Threshold of promoters	0.20
Length of the sequence in bp	400

Further analysis revealed that all *pgrB* isolates have a part of the gene which is identical to *pgrA* isolates which then diverge to *pgrB* suggesting the mosaic structure of *pgrB*. The divergence of the sequence to *pgrB* may indicate an evolutionary change in the gene. In all *pgrB* strains, the variation in the sequences occurred in the region of approximately 600 bp from the site 582 – 1190 on the sequence alignment (Figure 4.7). In the region 2, all *pgrB* isolates from the UK and Australia except the isolates 03563250 and 03563366 contained 3 bp deletions of sequences at the site 1137 and 1147 on the sequence alignment. However, this deletion was not observed in any of the *pgrA* isolates (Figure 4.8).

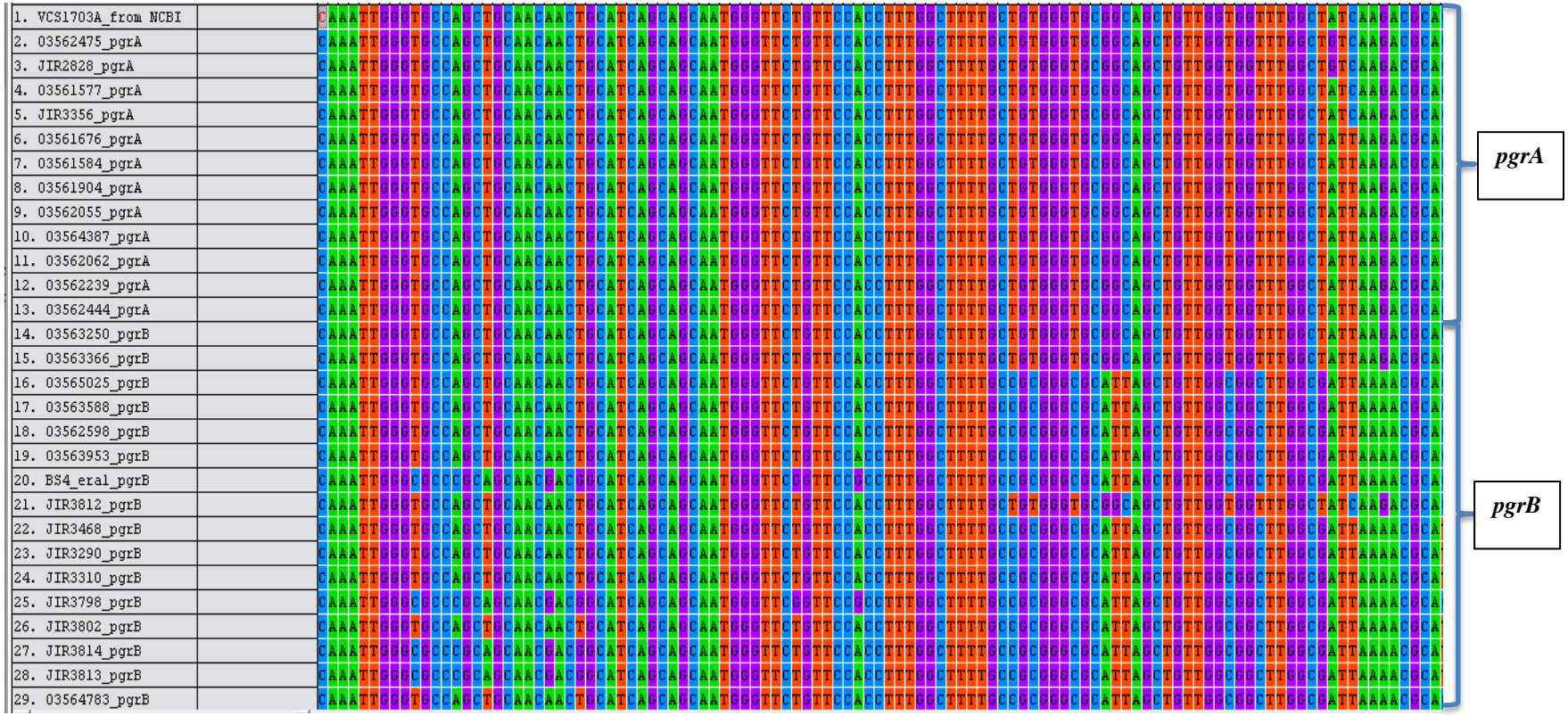


Figure 4.7: Divergence of sequence from *pgrA* to *pgrB*.

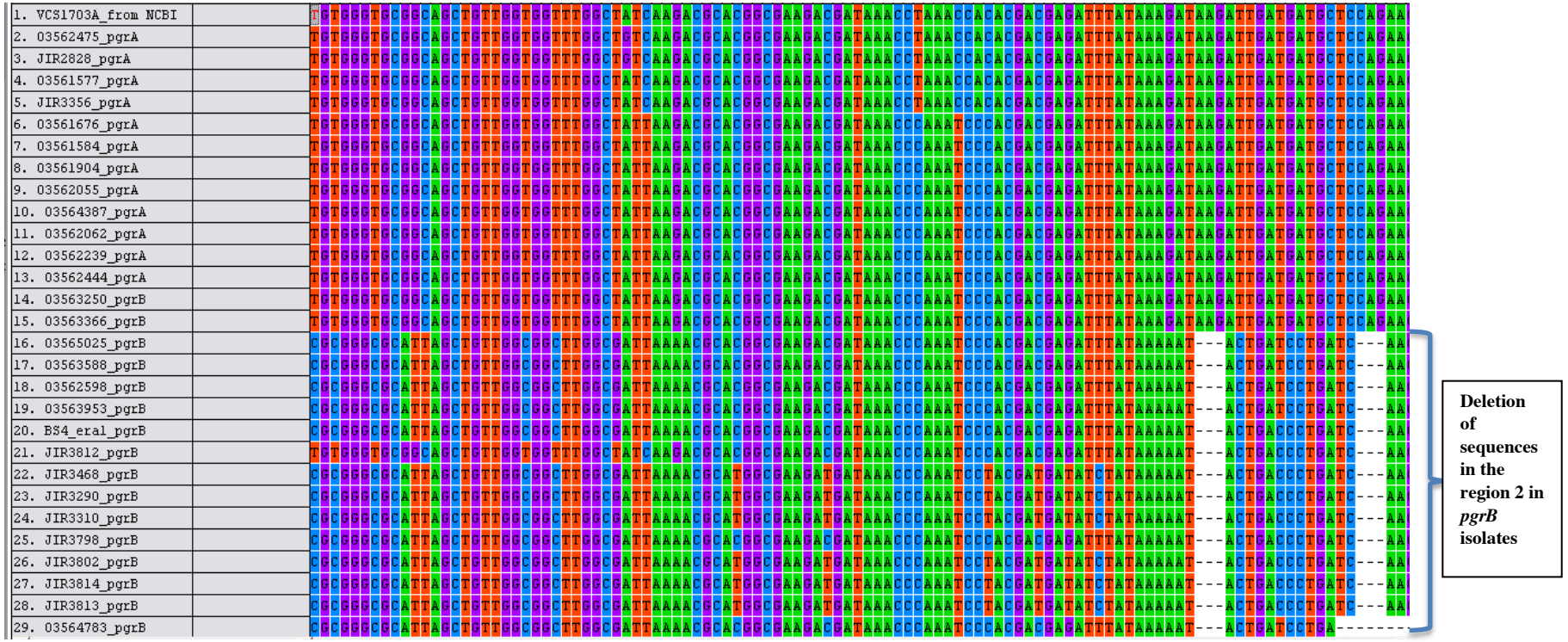


Figure 4.8: Deletions of sequences in the region 2 in *pgrB* isolates from the UK and Australia.

4.4.5. Structure of PgrA.

The structure of PgrA was predicted using the crystal structure of the extracellular lipase lipa from *Serratia marcescens* with confidence estimated at 98.8 %. PgrA contained a helix-turn-helix structural domain and two beta pleated sheets. Three distinct regions, from the N-terminus ASN 746 disordered region was observed up to THR 776, followed by two beta pleated sheets (A: SER859 to GLU 972, B; GLY 984 to SER 1094 conjoined with a short linker (Figure 4.9, Appendix 7).

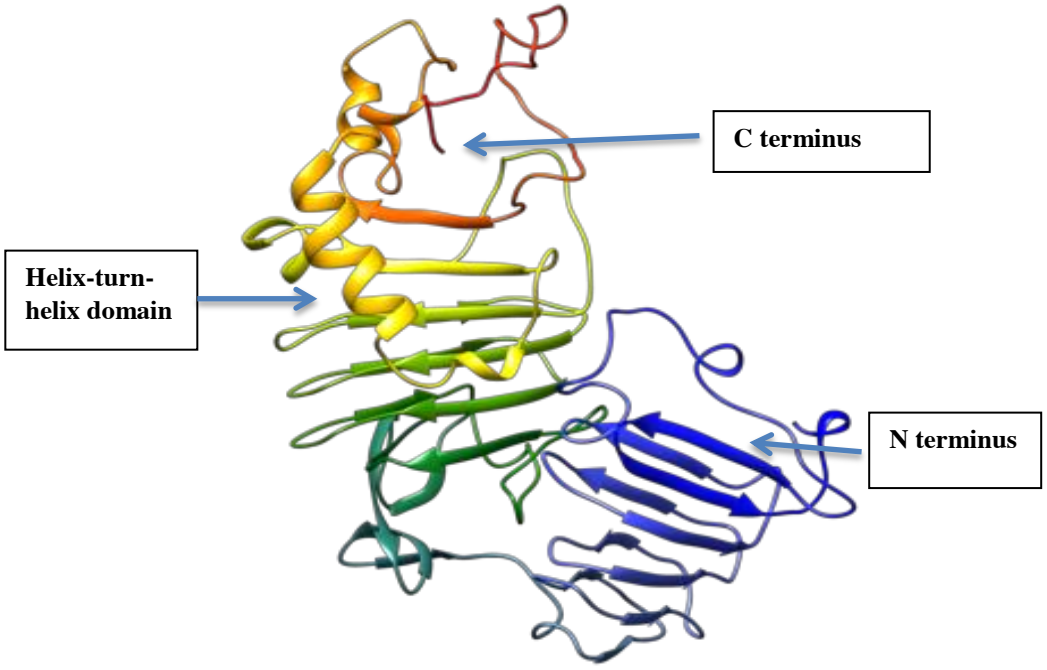


Figure 4.9: Predicted structure of PgrA. Blue colour indicates N terminus and Red indicates C terminus of the protein. The structure contains a helix-turn-helix domain and two beta pleated sheets (Red and Green). The structure does not take into account the number of tandem repeats present in R1 and R2 region in PgrA.

Further analysis indicated no obvious transmembrane regions; which were evident on the model. The polar residues are interdispersed between the hydrophobic residues such

that there is no clear obvious region where the protein could span the lipid membrane. The predicted structure was rotated 90 degrees and surface representation with hydrophobicity (grey) and polar residues (blue) showed no distinct regions to indicate transmembrane localisation sites on the structure (Figure 4.10 A, B, C and D). However, it should be noted that the model does contain the whole amino acid sequence. The tandem repeats were not included in this model; homology modelling from Phyre2 and SwissModel prediction servers could not identify known motifs for such sequences, regardless of the presence or absence of the repeats.

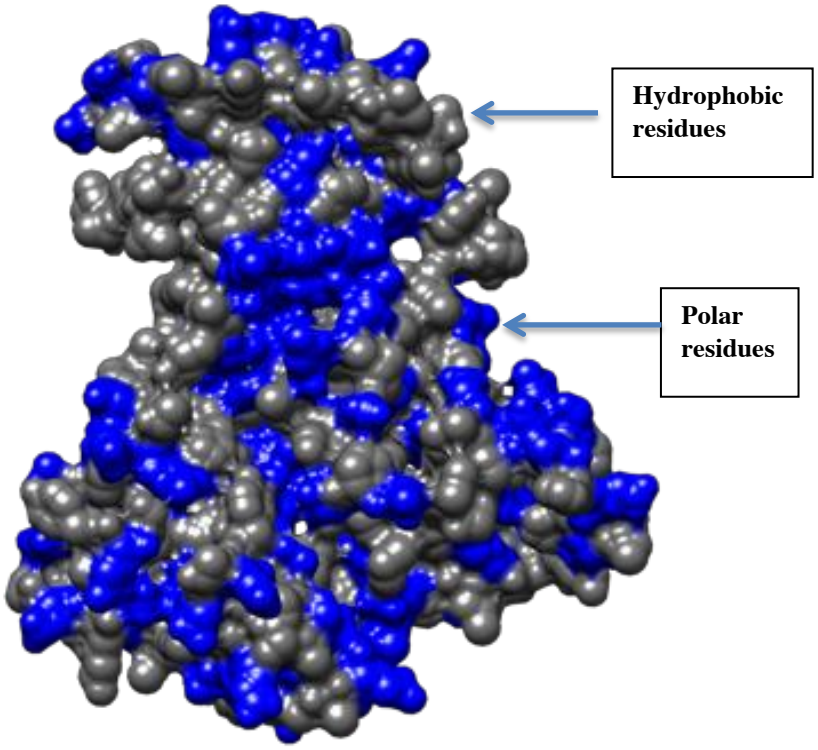


Figure 4.10 A: Surface representation (at 90 degrees) of PgrA protein with polar residues coloured in blue and hydrophobic residues in grey. No obvious transmembrane domains observed.

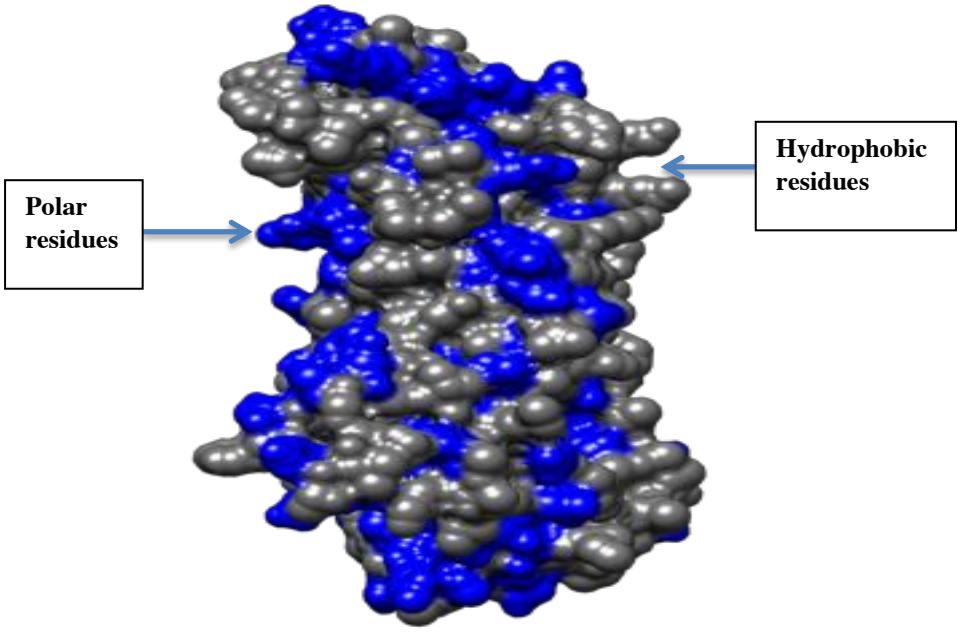


Figure 4.10 B: Surface representation (at 180 degrees) of PgrA protein with polar residues coloured in blue and hydrophobic residues in grey. No obvious transmembrane domains observed.

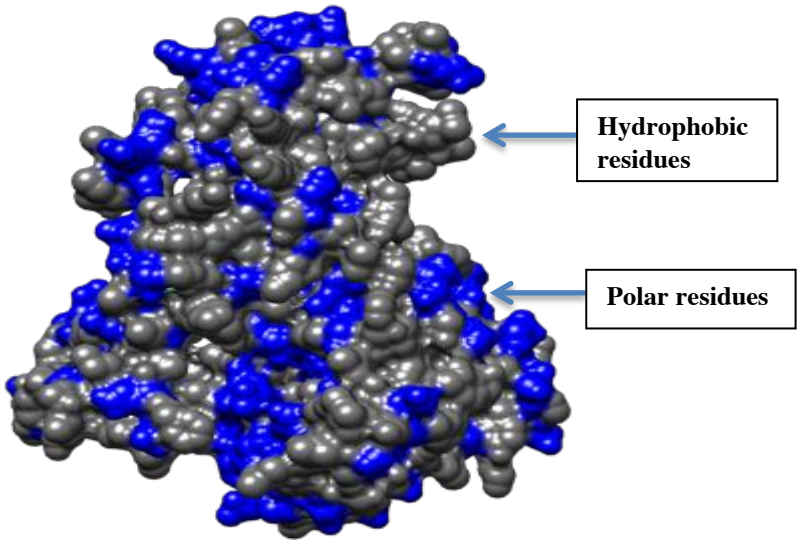


Figure 4.10 C: Surface representation (at 270 degrees) of PgrA protein with polar residues coloured in blue and hydrophobic residues in grey. No obvious transmembrane domains observed.

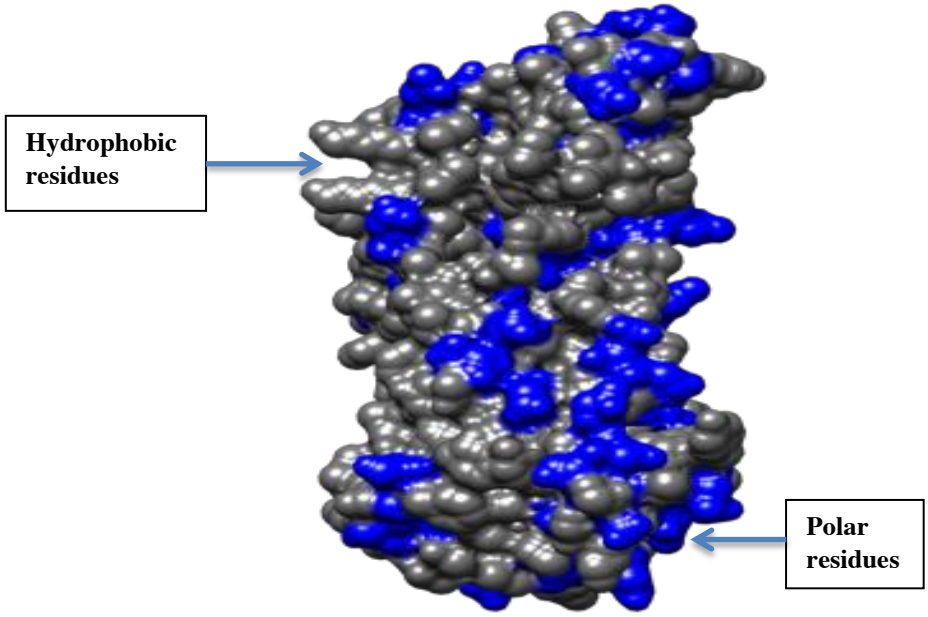


Figure 4.10 D: Surface representation (at 360 degrees) of PgrA protein with polar residues coloured in blue and hydrophobic residues in grey. No obvious transmembrane domains observed.

4.4.6. Expression of *pgrA* in strain VCS1703A.

The normalized levels of expression of *pgrA* showed a gradual increase from days 0 – 3, which decreased at day 8. The highest level of expression was observed at day 6 both in presence and absence of hoof horn (Figure 4.11). At day 6, *pgrA* expression increased 6 fold compared to day 3 in the presence of hoof horn. However, the expression increased only 4 fold when grown on non-hoof horn. Lower levels of expression were observed at day 8 compared to day 6 both in presence and absence of hoof horn and was estimated at 3 fold decrease in hoof horn and 2 fold decrease in non-hoof horn at day 8 (Figure 4.11). These results indicate that *pgrA* in the wild type strain VCS1703A was induced by hoof horn at day 6. The relative levels of expression were significantly higher ($p = 0.012$) with hoof horn than without at day 6 compared to other time points (Figure 4.11).

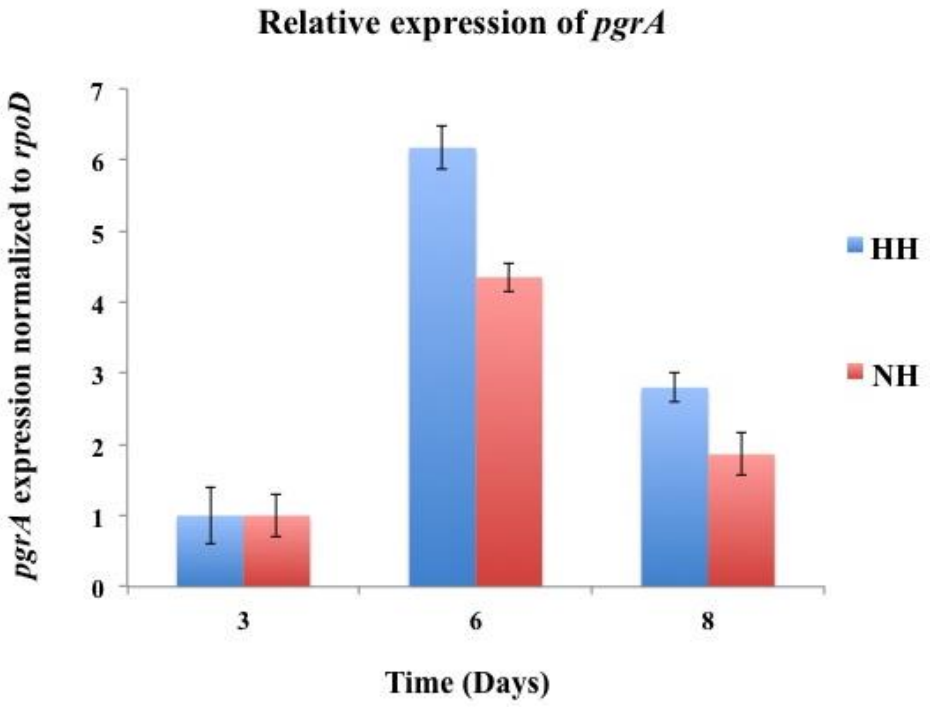


Figure 4.11: Expression of *pgrA* normalized to *rpoD* in *D. nodosus* strain VCS1703A grown with hoof horn and non-hoof horn. (A) Levels of expression of *pgrA* gene from the cDNA in *D. nodosus* strain VCS1703A. Blue bars indicate levels of expression in the presence of hoof horn (HH) and red bars indicate non-hoof horn (NH). Levels of expression of *pgrA* were normalized against *rpoD* from the cDNA in *D. nodosus* strain VCS1703A. (Error bars represent + and – standard deviation).

4.4.7. Expression of *pgrB* in strain BS4.

The normalized levels of expression of *pgrB* were constant at day 6 relative to day 3. However, the expression increased at day 8 both with and without hoof-horn. At day 6, the levels of expression were just above 1 with hoof horn compared to day 3 whereas with non-hoof, the levels were constant relative to day 3. At day 8, there was 2.5-fold increase in the expression compared to days 3 and 6 (Figure 4.12). No significant differences were observed in *pgrB* transcript levels both in presence and absence of

hoof-horn at each time point indicating that *pgrB* in the benign strain BS4 was not induced by hoof horn *in vitro*.

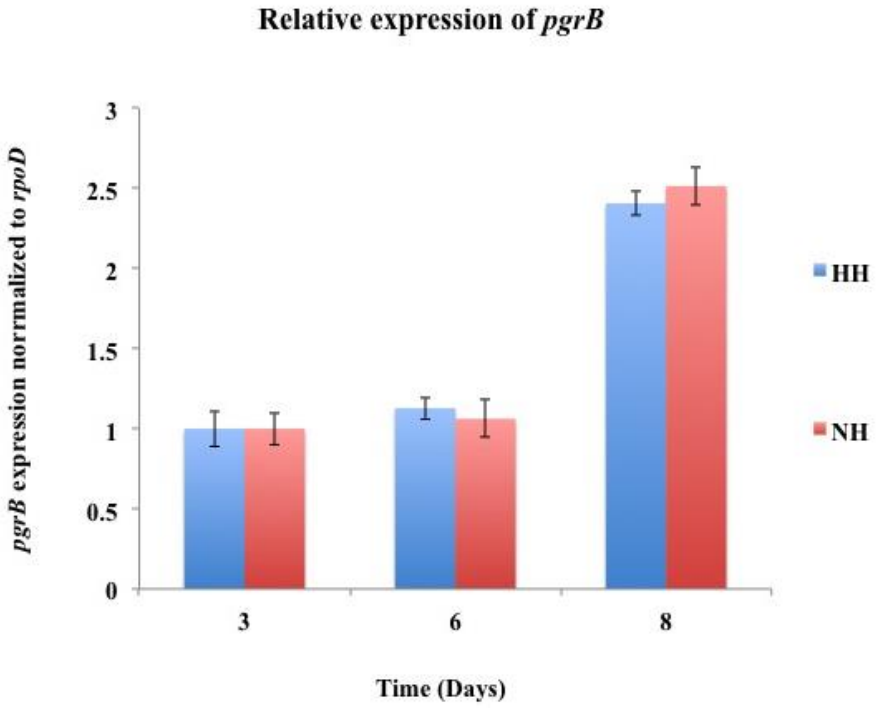


Figure 4.12: Expression of *pgrB* normalized to *rpoD* in *D. nodosus* strain BS4 grown with hoof horn and non-hoof horn. (A) Levels of expression of *pgrB* gene from the cDNA in *D. nodosus* strain BS4. Blue bars indicate levels of expression in the presence of hoof horn (HH) and red bars indicate non-hoof horn (NH). Levels of expression of *pgrB* were normalized against housekeeping gene *rpoD* from the cDNA in *D. nodosus* strain BS4 (Error bars represent + and – standard deviation).

4.5. Discussion.

This is the first study to describe the genetic relationship between *D. nodosus* isolates from UK sheep and a large collection of Australian and Scandinavian strains isolated from sheep in their respective countries. The aim was to compare the genomes of UK

isolates with those already sequenced by the group in Melbourne (Kennan, 2014). The study formed part of a collaboration with the University of Monash between Professors Wellington, Green and Rood and as a result of a further travelling scholarship, some of the comparative work was performed in the Monash labs under the supervision of Professor Rood. The aim was to determine the relatedness of the UK isolates to a large group of Australian strains. A detailed study have already been completed between the Australian and Scandinavian strains and provided the evidence that the isolates on the basis of SNP analysis divided into two main clades, clade I and clade II. A case was made to support the hypothesis that clade I contained predominantly virulent strains of *D*. *nodosus* with clade II comprised of mainly benign strains. The key genotype correlating with this bifurcation was the protease *aprV2* belonging to the virulent clade whereas clade II contained mostly *aprB2* homologs. The underlying hypothesis in this thesis has been that *D*. *nodosus* population on the feet of UK sheep are mixed and show a remarkable degree of diversity (Muzafar et al., 2015). A key component of this is the hypothesis that a further allele *pgr* is also an indicative gene with two homologs *pgrA* in virulent strains and *pgrB* in benign (Calvo-Bado et al., 2011a). To explore the *pgr* further, an additional aim was to study expression of this gene under different condition particularly those conducive to the induction of virulence determinants.

Genome analysis of the UK strains provided evidence on the basis of SNP characterization that all strains were recovered in clade I. This included the strain BS4 (*pgrB*), which is a known benign strain from the UK because it has a phenotype characterized by the protease thermostability test (Calvo-Bado et al., 2011a) and the

genotype *aprB2*. This is an exception to the existing classification of these strains into clades I and II as reported (Kennan, 2014), although in that study, a strain (RBG-17-BT-B-R) from Bhutan, with the presence of *aprB2*, also clustered into Clade I.

The widespread, even distribution of differences were observed across the entire bacterial chromosome of the UK strains indicating a substantial genetic distance between the isolates. *D. nodosus* strains from the UK formed two separate clusters within the clade I despite having the same size of the chromosome (approximately 1.39 MB). This observation suggests that there are two clonal populations of *D. nodosus* present on the same farm. This is surprising given the proximity of these isolates (taken from the same hoof in some instances, albeit at different times, but certainly on the same farm) and the observation that genetic exchange between isolates are likely to occur regularly. This would lead to the expectation that genome sequences would converge over time when co-existing in the same environment. In this study, we observe a second example of where two genetic types of *D. nodosus* exist in the same environment, but maintain distinct genetic identities. Similar observations were reported by Kennan et al. (Kennan, 2014).

In the current study, only 4.13% of the isolates could be recovered as the majority became unculturable after one transfer (Andrew Gilbert, MSc thesis). In addition one isolate apparently was re-isolated multiple times as established by MLVA; this strain was *pgrB* and *aprV2*. The MLVA typing groups strains below the species level and therefore the frequently occurring isolate with MLVA type 66 was recovered in both cluster α and β in clade I. The strains used in the current study were a mix of *pgrA*

(59%) and *pgrB* (41%). We hypothesized that *pgr* plays a role in virulence but it was clear that there was no correlation with *aprV2* and the classification into clades by Kennan et al. The separation of the isolates into two main clusters, α and β, in clade I was based on *pgrA*/*pgrB* status of the given isolate. However, *pgr* does not follow our hypothesis and the isolates appeared to be equally distributed between the two clusters with 86% *pgrA* and 14% *pgrB* present within the cluster α and 85% *pgrA* and 15% *pgrB* within the cluster β. The genome study by (Kennan et al., 2014) also reported the distribution of isolates into clades I and II irrespective whether the strains were *pgrA*/*pgrB*

The data presented in Fig. 5 in Kennan et al (2014) clearly shows the highly variable regions including VRL (virulence related locus), HsdS (type 1 restriction and modification system), VAP (virulence associated protein) 1 and 2, RTX (repeats in toxin, toxin proteins containing repeated glycine at C-terminal secreted via type I secretion system), OMP (outer membrane protein) 1, Bacteriophage (presence of bacteriophage genome in a number of benign strains and an integrated bacteriophage similar to VCS1703A in one virulent strain) and PGR (protein glycine repeat protein). These regions also could not be assembled in the UK strains presumably due to a combination of high variability tandem repeats. The sequence from the R1 variable region which is common in both variants was obtained by Sanger sequencing. *D*. *nodosus* strains (*pgrA*) from various geographical locations including the strains used in the current study and strains taken from the previous study by Calvo-Bado et al (Calvo-Bado et al., 2011a), clustered separately (Figure 4.5). A similar trend was observed for

pgrB strains. These observations confirm the suggestion that two distinct populations of *D*. *nodosus* co-exist in the same environment.

It was hypothesized that *pgrA* coded for a virulence factor and that the two transmembrane helices were responsible for colonization of *D*. *nodosus* to the foot (Calvo-Bado et al., 2011a). An infection study by Kennan et at (2010) reported that protease AprV2 was an essential virulence factor of *D*. *nodosus* and that the sheep infected with the wild type strain VCS1703A had intact AprV2 developed virulent footrot (Kennan et al., 2010). However, the mutants of *aprV2* did not cause the disease and their protease activity were significantly reduced than the wild type strain. An in vitro study was performed to investigate the potential for *pgr* to serve as a virulence factor and analyse *pgrA*/*B* expression using hoof horn as a stimulant. The findings indicated that *pgrA* might be involved in the colonization of *D*. *nodosus* to the foot but its even distribution within the clades I and II suggest that it does not have a major role in the virulence of *D*. *nodosus*. Clearly the conditions conducive to the expression of PgrB were not produced in the experiments, so either it is a protein with a diverse function compared to the PgrA or it may be a possible pseudogene which is an allele that is never expressed and has a range of deletions. The studies in other Gram negative bacteria such as *Yersinia* and *Vibrio* species reported the presence of pseudogenes within their respective genomes (Lerat and Ochman, 2005), and this genome reduction is a characteristic of pathogens and results in small genomes such as that seen in *D*. *nodosus*.

In the current study, it was determined that both *pgrA* and *pgrB* isolates from various geographical locations have the highly conserved upstream region suggesting a common origin. The divergence in *pgrB* downstream of this region may be due to recombination events as *D*. *nodosus* is highly transformable and could evolve new genes via transformation (Kennan et al., 2003; Verstrepen et al., 2005) and result in the formation of gene mosaics. The latter can occur in bacteria as a result of recombination events following the horizontal gene transfer (Smith, 1996; Smith et al., 1991). Gene mosaics have also been reported in *Neisseria* species making them less susceptible to oral Cephalosporins (Ochiai et al., 2008a). Other reports include mosaic structures in the polymorphic protein PspA of the human pathogen *Streptococcus pneumonia* (Hollingshead et al., 2000). *pgrA* and *pgrB* possess a number of tandem repeats, thus, making them polymorphic. This is commonly seen in proteins that are under constant selection against the immune system as reported for *Staphylococcus aureus* protein A (Kobayashi and DeLeo, 2013) .

PgrA protein contains glycine and proline rich repeats in R1 and R2 regions respectively. The latter is hydrophobic and imparts rigidity to the structure of the protein and the former makes the protein flexible, thus aiding in the secondary structure. A previous study by Calvo-Bado et al (2011) suggested that PgrA may be an outer membrane protein and possess the characteristic helix-turn-helix with two transmembrane domains that may have a role in attachment of *D*. *nodosus* to the foot (Calvo-Bado et al., 2011a). However, in the current study, the predicted structure of PgrA did not indicate any alpha helices. Therefore, further work is required to determine

the structure of PgrA and if it contains any transmembrane domains. However, the collagen like cell surface motifs were present in all PgrA*'s* suggesting an involvement with collagen. Further work will help in understanding the role of the PgrA in footrot disease. In addition, more studies are needed to determine if *pgrB* can be expressed under different conditions and is a fully functional protein.

Chapter 5

General Discussion

Diseases of domesticated livestock pose serious risk to health and welfare of the animals and effective control of such diseases is pivotal for health and productivity. It is essential to study the animal movements and risk associated with them to understand disease epidemiology which will aid in the control of the disease. The spread of infectious diseases is dependent on the movements of domestic and wild animals and transmission occurs by direct contact and through vectors, aerosols and the environment. Movements of livestock have resulted in the spread of diseases that are harmful to animals and may also pose a threat to human life. Some examples of these diseases include footrot, bovine tuberculosis, foot and mouth disease, rabies, rinderpest, Newcastle disease and have significant impact on domestic livestock population, wildlife and economy of the country (Bengis et al., 2002; Fevre et al., 2006). However, wild life can be a potent source of disease as in the case of bovine TB where European badgers act as a reservoir of infection (Waters et al., 2012). Recent studies have reported the occurrence of *Mycobacterium bovis* in sheep, pigs (Bailey et al., 2013) and deer (Delahay et al., 2002) in the UK and Republic of Ireland.

The environment can also harbor disease which may be shed by wild life or farm animals and a number of studies have been done to provide detailed evidence of the existence of the potential for environmental transfer from one animal to another. Examples of such diseases include bovine tuberculosis, paratuberculosis (Windsor, 2015), Brucellosis (Pappas et al., 2006).

A number of diseases of sheep cause serious production losses and includes footrot (Wassink et al., 2010), mastitis caused by *Staphylococcus aureus* (Mavrogianni et al., 2011), *Mycobacterium avium* sub species paratuberculosis (Nebbia et al., 2006). Such diseases are difficult to manage due to spread of the infectious agent during the managemental practices such as lambing and migration of herds to highland pasture during a specific time of the year. The mixing of the sheep can lead to transmission of virulent strains which may be a potential cause for a disease outbreak.

Footrot is an infectious disease of sheep which causes serious economic losses to the countries worldwide and costing the UK up to £84 M annually (Nieuwhof and Bishop, 2005; Wassink et al., 2010a). The control of footrot is essential for animal welfare and productivity. Whilst previous studies have highlighted that infected sheep are the reservoir of infection (Green et al., 2007; Smith et al., 2014; Whittington, 1995), that *D*. *nodosus* may be present asymptomatically in a flock (Depiazzi et al., 1998) and in individual sheep in diseased flocks (Kaler et al., 2012), transmission is thought to occur indirectly via contaminated pasture (Beveridge, 1941). In the UK, *D*. *nodosus* has been detected in the soil where sheep are kept for grazing (Witcomb, 2012). Contamination of pasture is likely to occur following an outbreak of footrot and in the current study, *D*. *nodosus* was detected in soil, faecal balls within the interdigital space and fresh hoof prints further supporting the hypothesis that pasture is a potential source of *D*. *nodosus*. Previous studies have reported the occurrence of *D*. *nodosus* in the oral cavity of sheep (Witcomb, 2012). This may suggest another potential source as the gingival crevices can harbour bacteria and therefore a likelihood of transmission to newborn lambs during

physical contact between ewe and lamb.

The work presented in thesis highlights the environmental reservoirs of the footrot causal agent *D. nodosus*, spread of this bacterium between ewes and lambs, its ability to persist in soil and the relationship of UK isolates with the global *D. nodosus* strain population. One of the key aims of the project was to improve our understanding of the potential for *D. nodosus* transmission via the environment within a flock of ewes and lambs. Lambs are born free from infection but rapidly become colonized by *D. nodosus* as detected by the molecular markers *rpoD* and 16S rRNA gene. The likely source of colonization to lambs was straw bedding from the communal pens. The rapid contamination of *D. nodosus* in lambs has been reported in this thesis for the first time and this will have implications on the lambing management.

Lambs had higher population of *D. nodosus* on their feet 5-13 h after birth, which was estimated at 10^3 to 10^5 *rpoD* genome equivalents per swab. It is interesting how lambs acquired such a large *D. nodosus* population on their feet within 13 h of birth and almost certainly indicates direct transfer of *D. nodosus* via material such as faecal balls stuck within the interdigital space. This provides *D. nodosus* with the opportunity to colonize the lambs' feet during the days after birth. It must be appreciated that the molecular detection methods did not prove the establishment of a living population. However, the lambs monitored at birth were observed over four months and all ten lambs developed ID when put out to pasture. What this work clearly demonstrates is that *D. nodosus* is not transmitted vertically from ewes to their lambs prior to birth.

In the current study, none of the lambs were observed to develop footrot. This could be due to the presence of intact interdigital skin on the feet. In addition the lambs did not receive any injuries to their feet during grazing which could enable entry of *D*. *nodosus* and secondary bacteria into the epidermal matrix, thus leading to the development of footrot. Instead ID developed which is characterized by an inflammatory reaction to the presence of *D*. *nodosus*, which may be producing proteases and other enzymes causing inflammation. However, it has been postulated that *D*. *nodosus* can stay in the hoof in a dormant state during dry conditions and when the conditions become favourable, the disease progression occurs (Beveridge, 1941; Mohler and Washburn, 1905). A number of studies have reported *D*. *nodosus* on the feet of apparently healthy sheep with no signs of disease (Calvo-Bado et al., 2011b; Moore, 2005; Witcomb, 2012). *D*. *nodosus* is capable of causing footrot only when the damage to the epidermis of the interdigital skin had occurred which caused a shift in the microbial community of the apparently healthy foot, thus increasing the incidence of the disease (Calvo-Bado et al., 2011b; Kaler et al., 2010a; Kaler et al., 2010b; Roberts and Egerton, 1969).

Ewes and lambs described in Chapter 2 were observed to have a mixed population of *D*. *nodosus* as indicated by diverse *pgrA* with varying tandem repeats in the R1 region. The diversity of strains observed on individual feet has been also reported in previous studies (Claxton et al., 1983; Jelinek et al., 2000; Moore et al., 2005a). A number of papers have noted the use of molecular and phenotypic markers as tools for virulence and forensic epidemiology and included *fimA*, elastase and protease thermostability test (Palmer, 1993; Stewart, 1979; Zhou and Hickford, 2000), but there are reports of false positives

for the thermostability test (Cheetham et al., 2006). Previous studies have sought to produce further markers of virulence (Calvo-Bado et al., 2011a) but only the work done by (Kennan et al., 2010) provided definitive data on a sole molecular marker *aprV2* which has replaced the phenotypic thermostability test for establishing virulence

The strain with MLVA type 66 was isolated from five lambs with the remaining five showing 18 MLVA types. This means that strain type 66 was prevalent on the foot and/or outcompeted other strains during the isolation process. It may be possible that the MLVA type 66 was sampled more frequently during second swabbing of the infected area but cultivation swabs were always done first followed by swabs for molecular analysis. Several studies have reported swabs as a commonly used method for sampling the interdigital skin (Bennett et al., 2009; Hill et al., 2010) and it is likely that the first swab will contain more bacteria than the subsequent swabs of the same area. Similar studies were performed in humans and reported that first swab contained ten times more bacteria than the subsequent swabs of the infected area (Chamberlain et al., 1997). The strains with MLVA type 66 may have the potential for causing an outbreak of footrot in a healthy flock. However, further studies should be performed to determine the fitness, ease of cultivation and infectivity of the MLVA type 66 strains in the occurrence of virulent footrot.

D. *nodosus* was detected in a range of environmental samples, its persistence in the environment is of the utmost importance for the management of disease on a farm. The work reported in Chapter 3 demonstrated that viable *D*. *nodosus* can be detected for up

to 30 days in four contrasting soils with larger populations surviving in clay soil. These findings are novel and survival was noted for a substantially longer period than previously reported. A next stage would be to investigate whether persistence of *D. nodosus* in soil from pasture is of this duration. This information would then be useful to provide farmers with guidelines for new control strategies to protect sheep from exposure to the pathogen on farmland. The survival in the presence of oxygen could be attributed to effective free radical control possibly by the presence of enzymes coping with oxidative stress such as superoxide dismutase and genes for aerobic metabolic pathways including ubiquinone biosynthesis, ribonucleotide reductase and cytochrome oxidase suggesting that *D. nodosus* might grow microaerophilically (Myers et al., 2007). During genome reduction, *D. nodosus* has lost a significant biosynthetic capability and now requires supply of essential amino acids for growth. This feature is observed in organisms with small genomes such as *Buchnera* species (Shigenobu et al., 2000). *D. nodosus* requires specific aminoacids for its growth as it is incapable of growing on carbohydrates, thus relying on fermentation of aminoacids as a carbon and energy source (Rood, 2005). *D. nodosus* was able to survive and remain viable aerobically for at least 10 days on the growth media. This may suggest that *D. nodosus* remains viable after exposure to oxygen in the hoof and outside of the host. It is hypothesized here that *D. nodosus* does not grow in the soil, but can survive within the clay lattices under very dry conditions. No evidence was found that *D. nodosus* could grow in the soil and this would not be expected due to its exacting nutritional requirements which relate to loss of the reductive evolution and the loss of key biosynthetic pathways resulting in the small genome (Myers et al., 2007). The surviving populations in clay soil can be

explained by higher buffering capacity, water retention and better nutrient availability of the clay soils (Bitton et al., 1972; England et al., 1993; Heynen et al., 1988). The findings reported in Chapter 3 are substantially longer than the current recommendation of resting a field for 14 days to stop onward transmission (Beveridge, 1941; Whittington and Nicholls, 1995). However, survival of an infectious dose for 14 days has been determined under the Australian conditions and depends on the bacterial strain, soil type and climate of the region (Egerton et al., 1969). It should be noted that the soil experiments were carried in the micrososms in the laboratory and further research is needed to determine the precise condition affecting the *D*. *nodosus* survival in soil under field conditions in the UK.

In the current study using WGS it was observed that two main genetic types (clusters α and β) of *D*. *nodosus* existed on the same foot in an individual sheep both at the same and different times. This would lead to an expectation that genome sequences may converge over time when co-existing in the same environment. Analysis of previously used molecular markers such as *fimA* indicated a correlation with these cluster groupings. However, what was striking from the WGS data was the low variability across the whole genome for the UK isolates compared to those sequenced from Australia (Kennan et al., 2014).

In addition, MLVA provided a similar approach to segregating diverse strains (Russell et al., 2014), although for isolates in the current study, this method showed a lot of diversity within SNP clusters which also had isolates with variable *pgr* status and

serogroups. It is not clear what factors are responsible for the genetic changes that occur in *D. nodosus* strains. A number of factors such as prolonged infection and time of treatment in regard to the stage of disease have been reported to affect the rate of genetic diversity in *Mycobacterium tuberculosis* in humans (Niemann et al., 1999; Warren et al., 2002). There is another possibility that host's selective immune pressure may be responsible for the occurrence of genetic diversity in *D. nodosus*. The polymorphism observed in *pgr* gene was hypothesized to occur due to selective immune pressure from the host (Calvo-Bado et al., 2011a). Other studies have reported recombination rather than point mutation responsible for genetic variation as observed in outer membrane proteins that are under strong selective pressure (Feil and Enright, 2004).

All *D. nodosus* strains isolated from the foot swabs were virulent based on the presence of thermostable protease *aprV2* and no strains containing the benign genotype *aprB2* were isolated during the current study. Whole genome analysis of a selection of 22 cohort isolates obtained from swabs during the year of study provided further evidence for virulent genotype as they clustered in clade I, as defined by Kennan et al (2014) Clade I contained uniformly virulent strains all containing *aprV2* (Kennan et al., 2014). A known benign strain BS4 from the UK also grouped into clade I but was found to contain *aprB2*, which does not correspond with the consensus of other strains in this group. Whole genome analysis and SNP estimation was used to delineate the clades containing strains from predominantly Australia and Scandinavia (n=99), with a limited number of representatives from other countries including one strain, 153-LB2 from the UK also recovered in clade I (Kennan et al., 2014). An isolate recovered from a flock in

Bhutan (RBG-17-BT-B-R) also contained *aprB2* but grouped in clade I. Therefore, less than 2% of the strains characterized in the Australian study and the current study failed to follow the consensus of benign *aprB2* genotype in clade II. The SNP analysis further substantiated the use of this protease gene to define the benign genotype, although 1.6% of strains were exceptional. It remains to be established if a more comprehensive study of UK strains follow the clade I grouping. Thus for the UK, further work is needed on the utility of *aprV2*/*B2* as a diagnostic tool for establishing virulent or benign status. If further analysis confirms that strains classified as benign are truly benign, then a biological control strategy could be proposed for regions like the UK where multiple serogroups have precluded effective vaccine development. Lambs' feet could be sprayed with a known benign strain immediately after birth, which will competitively exclude further colonization by virulent strains. On the UK farms, this can be achieved by spraying lamb's feet at birth with the benign strain BS4, detailed studies would be required to establish if this is feasible and competitive as an effective strategy. A number of studies have used competitive exclusion; it has been reported in poultry where inoculation with *Bacillus subtilis* spores was used to competitively exclude an *E. coli* virulent strain O78:K80 in the gut (La Ragione et al., 2001).

The isolates used for WGS were a mix of *pgrA* and *pgrB* and it was hypothesized that *pgr* is a virulence factor [further supported by the work of Gilhuus et al (2014)] and both clusters α and β should contain *pgrA* but the alleles were equally distributed between the two clusters. A previous study also reported *pgrA* and *pgrB* to be randomly distributed between virulent and benign clades (Kennan et al., 2014). The current study has not

provided evidence for the association of *pgrA* genotype with virulence but this gene may be involved with attachment of *D*. *nodosus* to the foot which has also been reported in other studies (Calvo-Bado et al., 2011a; Gilhuus et al., 2014; Myers et al., 2007). The role played by *pgr* was further studied by measuring expression during exposure to hoof horn in the cultivation media. Significantly higher levels of expression occurred in the presence of hoof horn compared with other sources of nutrient. Previous work by Yvonne Stoltz (Stoltz, 2013) using linear and circular dichroism reported the interaction of PgrA with collagen. This evidence and the expression work suggest a role for the protein in attachment/colonization to tissues in the foot. In addition hoof horn showed no effect on the expression of *pgrB*. Thus it appears that two alleles existed in the population but these do not correlate with the two distinct clonal populations. *pgrB* is too distinct to be a pseudogene but recombination may have increased the diversity in the coding region. Pseudogenes are reported in Gram negative bacteria such as *Yersinia* and *Vibrio* species resulting in a reduced transcriptome and further genome reduction (Lerat and Ochman, 2005) and this may well be occurring in *D*. *nodosus* which has a small genome of approximately 1.39 Mb and has obviously undergone reductive evolution (Myers et al., 2007). There is evidence of extensive genome reduction due to the presence of pseudogenes in intracellular pathogens with small genomes such as *Rickettsia species* and *Mycobacterium* species (Moran, 2002). Other studies have reported the occurrence of pseudogenes in bacteria due to ongoing mutational processes within the genomes and in *Salmonella* species, psuedogenes are silent or have a low levels of expression (Kuo and Ochman, 2010).

The current study has demonstrated that the two alleles, *pgrA* and *pgrB* might have a common origin as indicated by the presence of highly conserved upstream region in both. However, *pgrB* showed divergence in the sequence downstream to the conserved region suggesting the occurrence of recombination events and transformation giving rise to gene mosaics. Previous studies have also suggested that evolution of new genes in *D. nodosus* could occur via transformation (Kennan et al., 2003; Verstrepen et al., 2005). A number of studies have reported that recombination events following horizontal gene transfer may be responsible for the occurrence of gene mosaics in bacteria (Smith, 1996). Other studies have reported gene mosaics in Gram negative bacteria *Neisseria* species which makes them less susceptible to oral antibiotics (Ochiai et al., 2008b) and polymorphic protein PspA in *Streptococcus pneumonia* in humans (Hollingshead et al., 2000).

Conclusion and future perspectives:

Conclusions:

The key novel findings from this thesis are listed below:

1. *D. nodosus* does not transmit vertically from ewes to the newborn lambs. Lambs are born free from *D. nodosus* and acquire the pathogen on their feet within the first 5 h – 13 h of birth.
2. Lambs' feet are colonized by multiple strains of *D. nodosus* as indicated by the presence *pgrA* with varying number of tandem repeats and multiple MLVA alleles present on the feet of their mothers or ewes sharing the same lambing pen.

3. Environmental samples such as straw bedding, soil balls within the interdigital space and contaminated faeces are the main source of *D. nodosus* to newborn lambs.
4. *D. nodosus* can survive in soil microcosms for at least 40 days with viable cells surviving for up to 30 days in different soils, especially clay soil.
5. A low temperature of 5 degrees was associated with a longer duration of survival than 25 degrees.
6. Comparative SNP analysis of global *D. nodosus* isolates indicate that UK isolates group into Clade I and are classified as virulent based on the presence of thermostable protease *aprV2*. However, a benign strain from the UK, BS4 with a thermolabile protease *aprB2* and *pgrB* also clusters into Clade I. An even distribution of *pgrA*/*B* was observed within individual clusters in Clade I.
7. *pgrA* was induced by hoof horn in media *in vitro* whereas hoof horn showed no effect on the expression of *pgrB*.

Future perspectives:

Since a number of strains were detected on the feet of lambs 5 – 13 h after birth, it would be interesting to follow lambs longitudinally to observe changes in the *D. nodosus* population and disease development over time. Further studies should be conducted with a known benign strain (*pgrB*) for use as biological control. Biological control has potential for devising new measures for the control of virulent footrot not only in the UK but worldwide. More population genetics in situ and understanding of the role of *pgr* in the pathogenesis should be conducted.

A known virulent strain of *D*. *nodosus* (VCS1703A) has been reported to fulfill molecular Koch's postulates. This strain has a *pgrA* type gene and is able to cause disease in sheep. Therefore, further research should be conducted to study the exoproteome of *D*. *nodosus* for determination of any transmembrane domains as a tool for attachment.

D. *nodosus* could survive longer than 40 days in soil. Therefore, further survival experiments may be set up for over 40 days with increased sampling points. This could be conducted in the laboratory and under field conditions. In the current study, a *D*. *nodosus* population of approximately 10^6 *rpoD* genome equivalents were detected at day 40. However, it is still unknown whether 10^6 *rpoD* genome equivalents are sufficient enough to cause footrot in sheep. In this context, infection studies should be conducted to determine the infectious dose of *D*. *nodosus* in sheep in the UK. Determination of infectious dose would have a major role in devising new control strategies for footrot in the UK.

BIBLIOGRAPHY

Abbott, K.A., Egerton, J.R., 2003. Effect of climatic region on the clinical expression of footrot of lesser clinical severity (intermediate footrot) in sheep. Australian veterinary journal 81, 756-762.

Abbott, K.A., Lewis, C.J., 2005. Current approaches to the management of ovine footrot. The Veterinary Journal 169, 28-41.

Arias, R., Nelson, S., Alvarez, A., 2000. Effect of Soil–matric Potential and Phylloplanes of Rotation-crops on the Survival of a Bioluminescent Xanthomonas campestris pv. campestris. European Journal of Plant Pathology 106, 109-116.

Artz, R.R.E., Avery, L.M., Jones, D.L., Killham, K., 2006. Potential pitfalls in the quantitative molecular detection of *Escherichia coli* O157:H7 in environmental matrices. Canadian Journal of Microbiology 52, 482-488.

Bailey S.S, Crawshaw T.R, Smith N.H., 2013. *Mycobacterium bovis* infection in domestic pigs in Great Britain. Veterinary Journal 198:391-7.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. Journal of microbiological methods 55, 541-555.

Belloy, L., Giacometti, M., Boujon, P., Waldvogel, A., 2007. Detection of *Dichelobacter nodosus* in wild ungulates (capra ibex ibex and ovis aries musimon) and domestic sheep suffering from foot rot using a two-step polymerase chain reaction. Journal of Wildlife Diseases 43, 82-88.

Bengis R.G, Kock R.A, Fischer J., 2002. Infectious animal diseases: the wildlife/livestock interface. Reverse Science Techonology 21:53-65.

Bennett, G., Hickford, J., Sedcole, R., Zhou, H., 2009. *Dichelobacter nodosus*, *Fusobacterium necrophorum* and the epidemiology of footrot. Anaerobe 15, 173-176.

Beveridge 1941. A transmissible disease due to infection with *Fusiformis nodusus* (n.sp.): Studies on its cause, Epidemiology, and Control. Bulletin of the Council of Scientific and Industrial Research, Australia (Australia), 1-58.

Beveridge, W.I.B., 1936. A study of *Spirochaeta penortha* (n. sp.) isolated from foot-rot in sheep. Australian Journal of Experimental Biological Medical Science 14, 307-318.

Bhat, M.A., Wani, S.A., Hussain, I., Magray, S.N., Muzafar, M., 2012. Identification of two new serotypes within serogroup B of *Dichelobacter nodosus*. Anaerobe 18, 91-95.

Billington, S.J., Johnston, J.L., Rood, J.I., 1996. Virulence regions and virulence factors of the ovine footrot pathogen, *Dichelobacter nodosus*. FEMS Microbiol Lett 145, 147-156.

Bitton, G., Henis, Y., Lahav, N., 1972. Effect of several clay minerals and humic acid on the survival of *Klebsiella aerogenes* exposed to ultraviolet irradiation. Appl Microbiol 23, 870-874.

Brown, 1892. Veterinary Journal 35, 180-247.

Bugrysheva, J.V., Godfrey, H.P., Schwartz, I., Cabello, F.C., 2011. Patterns and regulation of ribosomal RNA transcription in Borrelia burgdorferi. BMC Microbiology 11, 17-17.

Buller, N.B., Ashley, P., Palmer, M., Pitman, D., Richards, R.B., Hampson, D.J., 2010. Understanding the Molecular Epidemiology of the Footrot Pathogen *Dichelobacter nodosus* To Support Control and Eradication Programs. Journal of clinical microbiology 48, 877-882.

Bzymek, M., Lovett, S.T., 2001. Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. Proc Natl Acad Sci U S A 98, 8319-8325.

Calvo-Bado, L.A., Green, L.E., Medley, G.F., Ul-Hassan, A., Grogono-Thomas, R., Buller, N., Kaler, J., Russell, C.L., Kennan, R.M., Rood, J.I., Wellington, E.M.H., 2011a. Detection and diversity of a putative novel heterogeneous polymorphic proline-glycine repeat (Pgr) protein in the footrot pathogen *Dichelobacter nodosus*. Veterinary microbiology 147, 358-366.

Calvo-Bado, L.A., Oakley, B.B., Dowd, S.E., Green, L.E., Medley, G.F., Ul-Hassan, A., Bateman, V., Gaze, W., Witcomb, L., Grogono-Thomas, R., Kaler, J., Russell, C.L., Wellington, E.M., 2011b. Ovine pedomics: the first study of the ovine foot 16S rRNA-based microbiome. ISME J 5, 1426-1437.

Cederlof, S.E., Hansen, T., Klaas, I.C., Angen, O., 2013. An evaluation of the ability of *Dichelobacter nodosus* to survive in soil. Acta Vet Scand 55, 4.

Chamberlain, A.N., Halablab, M.A., Gould, D.J., Miles, R.J., 1997. Distribution of Bacteria on Hands and the Effectiveness of Brief and Thorough Decontamination Procedures Using Non-medicated Soap. Zentralblatt für Bakteriologie 285, 565-575.

Cheetham, B.F., Tanjung, L.R., Sutherland, M., Druitt, J., Green, G., McFarlane, J., Bailey, G.D., Seaman, J.T., Katz, M.E., 2006. Improved diagnosis of virulent ovine footrot using the intA gene. Veterinary microbiology 116, 166-174.

Clark, B.L., Stewart, D.J., Emery, D.L., 1985. The role of *Fusobacterium necrophorum* and Bacteroides melaninogenicus in the aetiology of interdigital necrobacillosis in cattle. Australian veterinary journal 62, 47-49.

Claxton, P.D., Ribeiro, L.A., Egerton, J.R., 1983. Classification of *Bacteroides nodosus* by agglutination tests. Australian veterinary journal 60, 331-334.

Collighan, R.J., Naylor, R.D., Martin, P.K., Cooley, B.A., Buller, N., Woodward, M.J., 2000. A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a Treponeme isolated from human periodontitis and bovine digital dermatitis. Veterinary microbiology 74, 249-257.

Collighan, R.J., Woodward, M.J., 1997. Spirochaetes and other bacterial species associated with bovine digital dermatitis. FEMS Microbiology Letters 156, 37-41.

Culman, S., Bukowski, R., Gauch, H., Cadillo-Quiroz, H., Buckley, D., 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics 10, 171.

D., S., 1979. The role of elastase in differentiation of *Bacteroides nodosus* infections in sheep and cattle. Research in Veterinary Science 27, 99-105.

Dalrymple, B., Mattick, J., 1987. An analysis of the organization and evolution of type 4 fimbrial (MePhe) subunit proteins. Journal of Molecular Evolution 25, 261-269.

Delahay R.J, De Leeuw A.N, Barlow A.M., 2002. The status of *Mycobacterium bovis* infection in UK wild mammals: a review. Veterinary Journal 164:90-105.

Demirkan, I., Carter, S.D., Winstanley, C., Bruce, K.D., Mcnair, N.M., Woodside, M., Hart, C.A., 2001. Isolation and characterisation of a novel spirochaete from severe virulent ovine foot rot. Journal of medical microbiology 50, 1061-1068.

Depiazzi, L.J., Henderson, J., Penhale, W.J., 1990. Measurement of protease thermostability, twitching motility and colony size of *Bacteroides nodosus*. Veterinary microbiology 22, 353-363.

Depiazzi, L.J., Roberts, W.D., Hawkins, C.D., Palmer, M.A., Pitman, D.R., McQuade, N.C., Jelinek, P.D., Devereaux, D.J., Rippon, R.J., 1998. Severity and persistence of footrot in Merino sheep experimentally infected with a protease thermostable strain of *Dichelobacter nodosus* at five sites. Aust Vet J 76, 32-38.

Dewhirst, F.E., Paster, B.J., La Fontaine, S., Rood, J.I., 1990. Transfer of Kingella indologenes (Snell and Lapage 1976) to the genus Suttonella gen. nov. as Suttonella indologenes comb. nov.; transfer of *Bacteroides nodosus* (Beveridge 1941) to the genus *Dichelobacter* gen. nov. as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter*, and *Suttonella* to *Cardiobacteriaceae* fam. nov. in the gamma division of Proteobacteria on the basis of 16S rRNA sequence comparisons. Int J Syst Bacteriol 40, 426-433.

Dhawi, A., Hart, C.A., Demirkan, I., Davies, I.H., Carter, S.D., 2005. Bovine digital dermatitis and severe virulent ovine foot rot: A common spirochaetal pathogenesis. The Veterinary Journal 169, 232-241.

Dhungyel, O., Schiller, N., Eppleston, J., Lehmann, D., Nilon, P., Ewers, A., Whittington, R., 2013. Outbreak-specific monovalent/bivalent vaccination to control and eradicate virulent ovine footrot. Vaccine 31, 1701-1706.

Dhungyel, O.P., Whittington, R.J., 2009. Modulation of inter-vaccination interval to avoid antigenic competition in multivalent footrot *(Dichelobacter nodosus)* vaccines in sheep. Vaccine 28, 470-473.

Dhungyel, O.P., Whittington, R.J., Ghimire, S.C., Egerton, J.R., 2001. Pilus ELISA and an anamnestic test for the diagnosis of virulent ovine footrot and its application in a disease control program in Nepal. Veterinary microbiology 79, 31-45.

Dice, L.R., 1945. Measures of the Amount of Ecologic Association Between Species". Ecology 26, 297-302.

Egerton, J.R., 1970. Successful vaccination of sheep against foot-rot. Australian veterinary journal 46, 114-115.

Egerton, J.R., 1973. Surface and somatic antigens of Fusiformis nodosus. J Comp Pathol 83, 151-159.

Egerton, J.R., 1974. Significance of *Fusiformis nodosus* serotypes in resistance of vaccinated sheep to experimental foot-rot. Australian veterinary journal 50, 59-62.

Egerton, J.R., Burrell, D.H., 1970. Prophylactic and therapeutic vaccination against ovine foot-rot. Australian veterinary journal 46, 517-522.

Egerton, J.R., Ghimire, S.C., Dhungyel, O.P., Shrestha, H.K., Joshi, H.D., Joshi, B.R., Abbott, K.A., Kristo, C., 2002. Eradication of virulent footrot from sheep and goats in an endemic area of Nepal and an evaluation of specific vaccination. The Veterinary record 151, 290-295.

Egerton, J.R., Laing, E.A., 1979. Characteristics of *Bacteroides nodosus* isolated from cattle. Veterinary microbiology 3, 269-279.

Egerton, J.R., Raadsma,H.W, 1991. Breeding Sheep for Resistance to Footrot. Breeding for Disease Resistance in Farm Animals. C.A.B. International, Wallingford, 347-370.

Egerton, J.R., Roberts, D.S., Parsonson, I.M., 1969. The aetiology and pathogenesis of ovine foot-rot. I. A histological study of the bacterial invasion. J Comp Pathol 79, 207-215.

Egerton, J.R., Seaman, J.T., Walker, R.I. 2004. Eradication of virulent footrot from New South Wales. In: Proceedings of the 13th International Symposium and 5th Conference on Lameness in Ruminants, Maribor, Slovenija, 11–15th February. In, 215–221.

Emery, D.L., Stewart, D.J., Clark, B.L., 1984. The comparative susceptibility of five breeds of sheep to foot-rot. Australian veterinary journal 61, 85-88.

England, L.S., Lee, H., Trevors, J.T., 1993. Bacterial survival in soil: Effect of clays and protozoa. Soil Biology and Biochemistry 25, 525-531.

Feil, E.J., Enright, M.C., 2004. Analyses of clonality and the evolution of bacterial pathogens. Current Opinion in Microbiology 7, 308-313.

Fevre E.M, Bronsvoort B.M, Hamilton K.A., 2006. Animal movements and the spread of infectious diseases. Trends Microbiology 14:125-31.

Fitzpatrick, J., Scott, M., Nolan, A., 2006. Assessment of pain and welfare in sheep. Small Ruminant Research 62, 55-61.

Fredricks, D.N., Fiedler, T.L., Thomas, K.K., Mitchell, C.M., Marrazzo, J.M., 2009. Changes in vaginal bacterial concentrations with intravaginal metronidazole therapy for bacterial vaginosis as assessed by quantitative PCR. J Clin Microbiol 47, 721-726.

Frisch, J.E., 1976. The comparative incidence of foot rot in bos taurus and bos indicus cattle. Australian veterinary journal 52, 228-229.

Frosth, S., König, U., Nyman, A.-K., Pringle, M., Aspán, A., 2015. Characterisation of *Dichelobacter nodosus* and detection of *Fusobacterium necrophorum* and *Treponema spp*. in sheep with different clinical manifestations of footrot. Veterinary microbiology 179, 82-90.

Ghimire, S.C., Egerton, J.R., Dhungyel, O.P., 1996. Characterisation of *Dichelobacter nodosus* isolated from footrot in sheep and goats in Nepal. Small Ruminant Research 23, 59-67.

Ghimire, S.C., Egerton, J.R., Dhungyel, O.P., Joshi, H.D., 1998. Identification and characterisation of serogroup M among Nepalese isolates of *Dichelobacter nodosus*, the transmitting agent of footrot in small ruminants. Veterinary microbiology 62, 217-233.

Gilbert, A., 2013. Analysing the Molecular, Bacteriological and Evolutionary Characteristics of *Dichelobacter nodosus* in a Single Flock of Sheep. M.Sc. University of Warwick ID: 1266252

Gilhuus, M., Kvitle, B., L'Abee-Lund, T.M., Vatn, S., Jorgensen, H.J., 2014. A recently introduced *Dichelobacter nodosus* strain caused an outbreak of footrot in Norway. Acta Vet Scand 56, 29.

Gilhuus, M., Vatn, S., Dhungyel, O.P., Tesfamichael, B., L'Abée-Lund, T.M., Jørgensen, H.J., 2013. Characterisation of *Dichelobacter nodosus* isolates from Norway. Veterinary microbiology 163, 142-148.

Glynn, T., 1993. Benign footrot--an epidemiological investigation into the occurrence, effects on production, response to treatment and influence of environmental factors. Australian veterinary journal 70, 7-12.

Gradin, J.L., Schmitz, J.A., 1977. Selective medium for isolation of *Bacteroides nodosus*. Journal of clinical microbiology 6, 298-302.

Gradin, J.L., Sonn, A. E., Bearwood, D. E., Schmitz, J. A., Smith, A. W. , 1991. Electron microscopic study of *Bacteroides nodosus* pili and associated structures. American Journal of Veterinary Research. 52, 206-211.

Graham, N.P., Egerton, J.R., 1968. Pathogenesis of ovine foot-rot: the role of some environmental factors. Australian veterinary journal 44, 235-240.

Green, L.E., George, T.R., 2008. Assessment of current knowledge of footrot in sheep with particular reference to *Dichelobacter nodosus* and implications for elimination or control strategies for sheep in Great Britain. Vet J 175, 173-180.

Green, L.E., Wassink, G.J., Grogono-Thomas, R., Moore, L.J., Medley, G.F., 2007. Looking after the individual to reduce disease in the flock: a binomial mixed effects model investigating the impact of individual sheep management of footrot and interdigital dermatitis in a prospective longitudinal study on one farm. Prev Vet Med 78, 172-178.

Gregory, T.S., 1939. Foot-rot in sheep. Australian veterinary journal 15, 160-167.

Grogono-Thomas, R., Johnston, A.M. 1997. A study of ovine lameness. MAFF Final Report. MAFF open contract OC59 45K.: DEFRA Publications: (London).

Grogono-Thomas, R., Wilsmore, A.J., Simon, A.J., Izzard, K.A., 1994. The use of long-acting oxytetracycline for the treatment of ovine footrot. British Veterinary Journal 150, 561-568.

Gurung, R.B., Dhungyel, O. P., Tshering, P., Egerton, J. R., 2006. The use of an autogenous *Dichelobacter nodosus* vaccine to eliminate clinical signs of virulent footrot in a sheep flock in Bhutan. Vet J 172, 356-363.

Han, X., Kennan, R.M., Davies, J.K., Reddacliff, L.A., Dhungyel, O.P., Whittington, R.J., Turnbull, L., Whitchurch, C.B., Rood, J.I., 2008. Twitching motility is essential for virulence in *Dichelobacter nodosus*. J Bacteriol 190, 3323-3335.

Han, X., Kennan, R.M., Parker, D., Davies, J.K., Rood, J.I., 2007. Type IV fimbrial biogenesis is required for protease secretion and natural transformation in *Dichelobacter nodosus*. J Bacteriol 189, 5022-5033.

Han, X., Kennan, R.M., Steer, D.L., Smith, A.I., Whisstock, J.C., Rood, J.I., 2012. The AprV5 subtilase is required for the optimal processing of all three extracellular serine proteases from *Dichelobacter nodosus*. PloS one 7, e47932.

Harriss, S.T., 1948. Proliferative Dermatitis of the Legs ("Strawberry Foot Rot") in Sheep. Journal of Comparative Pathology and Therapeutics 58, 314-328.

Heynen, C.E., Van Elsas, J.D., Kuikman, P.J., van Veen, J.A., 1988. Dynamics of Rhizobium leguminosarum biovar trifolii introduced into soil; the effect of bentonite clay on predation by protozoa. Soil Biology and Biochemistry 20, 483-488.

Hill, A.E., Dhungyel, O.P., Whittington, R.J., 2010. Diagnostic sampling strategies for virulent ovine footrot: Simulating detection of *Dichelobacter nodosus* serogroups for bivalent vaccine formulation. Preventive Veterinary Medicine 95, 127-136.

Hindmarsh, F., Fraser, J., 1985. Serogroups of *Bacteroides nodosus* isolated from ovine footrot in Britain. The Veterinary record 116, 187-188.

Hindmarsh, F., Fraser, J., Scott, K., 1989. Efficacy of a multivalent *Bacteroides nodosus* vaccine against foot rot in sheep in Britain. The Veterinary record 125, 128-130.

Hollingshead, S.K., Becker, R., Briles, D.E., 2000. Diversity of PspA: Mosaic Genes and Evidence for Past Recombination in *Streptococcus pneumoniae*. Infection and Immunity 68, 5889-5900.

Höper, H., Steinberg, C., Alabouvette, C., 1995. Involvement of clay type and pH in the mechanisms of soil suppressiveness to fusarium wilt of flax. Soil Biology and Biochemistry 27, 955-967.

Hunt, J.D., Brown, L.E., Wood, P.R., Stewart, D.J., Jackson, D.C., 1996. Manipulation of the helper T cell response to influence antigenic competition occurring with a multivalent vaccine. Immunol Cell Biol 74, 81-89.

Hunt, J.D., Jackson, D.C., Wood, P.R., Stewart, D.J., Brown, L.E., 1995. Immunological parameters associated with antigenic competition in a multivalent footrot vaccine. Vaccine 13, 1649-1657.

Ibekwe, A.M., Papiernik, S.K., Grieve, C.M., Yang, C.-H., 2011. Quantification of Persistence of *Escherichia coli* O157:H7 in Contrasting Soils. International Journal of Microbiology 2011.

Jaton, K., Bille, J., Greub, G., 2006. A novel real-time PCR to detect *Chlamydia trachomatis* in first-void urine or genital swabs. Journal of medical microbiology 55, 1667-1674.

Jelinek, P.D., Depiazzi, L.J., Galvin, D.A., Spicer, I.T., Palmer, M.A., Pitman, D.R., 2000. Occurrence of different strains of *Dichelobacter nodosus* in new clinical lesions in sheep exposed to footrot associated with multi-strain infections. Aust Vet J 78, 273-276.

Johnson, C.J., Phillips, K.E., Schramm, P.T., McKenzie, D., Aiken, J.M., Pedersen, J.A., 2006. Prions adhere to soil minerals and remain infectious. PLoS Pathog 2, e32.

Kaler, J., Daniels, S.L., Wright, J.L., Green, L.E., 2010a. Randomized clinical trial of long-acting oxytetracycline, foot trimming, and flunixine meglumine on time to recovery in sheep with footrot. J Vet Intern Med 24, 420-425.

Kaler, J., Green, L.E., 2008. Naming and recognition of six foot lesions of sheep using written and pictorial information: a study of 809 English sheep farmers. Prev Vet Med 83, 52-64.

Kaler, J., Green, L.E., 2009a. Farmers' practices and factors associated with the prevalence of all lameness and lameness attributed to interdigital dermatitis and footrot in sheep flocks in England in 2004. Prev Vet Med 92, 52-59.

Kaler, J., Green, L.E., 2009b. Farmers' practices and factors associated with the prevalence of all lameness and lameness attributed to interdigital dermatitis and footrot in sheep flocks in England in 2004. Preventive Veterinary Medicine 92, 52-59.

Kaler, J., Medley, G.F., Grogono-Thomas, R., Wellington, E.M.H., Calvo-Bado, L.A., Wassink, G.J., King, E.M., Moore, L.J., Russell, C., Green, L.E., 2010b. Factors associated with changes of state of foot conformation and lameness in a flock of sheep. Preventive Veterinary Medicine 97, 237-244.

Kaler, J., Wani, S.A., Hussain, I., Beg, S.A., Makhdoomi, M., Kabli, Z.A., Green, L.E., 2012. A clinical trial comparing parenteral oxytetracyline and enrofloxacin on time to recovery in sheep lame with acute or chronic footrot in Kashmir, India. BMC veterinary research 8, 12.

Katz, M.E., Howarth, P.M., Yong, W.K., Riffkin, G.G., Depiazzi, L.J., Rood, J.I., 1991. Identification of three gene regions associated with virulence in *Dichelobacter nodosus*, the causative agent of ovine footrot. J Gen Microbiol 137, 2117-2124.

KC, M., 1975. Clay mineralogy in relation to survival of soil bacteria. Annual Review Phytopathology 13, 357-373.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J.E., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protocols 10, 845-858.

Kennan, R.M., Dhungyel, O.P., Whittington, R.J., Egerton, J.R., Rood, J.I., 2001. The type IV fimbrial subunit gene (fimA) of *Dichelobacter nodosus* is essential for virulence, protease secretion, and natural competence. J Bacteriol 183, 4451-4458.

Kennan, R.M., Dhungyel, O.P., Whittington, R.J., Egerton, J.R., Rood, J.I., 2003. Transformation-mediated serogroup conversion of *Dichelobacter nodosus*. Vet Microbiol 92, 169-178.

Kennan, R.M., Gilhuus, M., Frosth, S., Seemann, T., Dhungyel, O.P., Whittington, R.J., Boyce, J.D., Powell, D.R., Aspán, A., Jørgensen, H.J., Bulach, D.M., Rood, J.I., 2014. Genomic Evidence for a Globally Distributed, Bimodal Population in the Ovine Footrot Pathogen Dichelobacter nodosus. mBio 5, e01821-01814.

Kennan, R.M., Gilhuus, M., Frosth, S. S., Seemann, T., Dhungyel, O. P., Whittington, R. J., Boyce, J. D., Powell, D. R., Aspán, A. J., Hannah J., Bulach, D. M., Rood, J. I., 2014. Genomic Evidence for a Globally Distributed, Bimodal Population in the Ovine Footrot Pathogen *Dichelobacter nodosus*. mBio 5, e01821-01814.

Kennan, R.M., Han, X., Porter, C.J., Rood, J.I., 2011. The pathogenesis of ovine footrot. Vet Microbiol 153, 59-66.

Kennan, R.M., Han, X., Porter, C. J., Rood, J. I., 2011. The pathogenesis of ovine footrot. Veterinary microbiology 153, 59-66.

Kennan, R.M., Wong, W., Dhungyel, O.P., Han, X., Wong, D., Parker, D., Rosado, C.J., Law, R.H., McGowan, S., Reeve, S.B., Levina, V., Powers, G.A., Pike, R.N., Bottomley, S.P., Smith, A.I., Marsh, I., Whittington, R.J., Whisstock, J.C., Porter, C.J., Rood, J.I., 2010. The subtilisin-like protease AprV2 is required for virulence and uses a novel disulphide-tethered exosite to bind substrates. PLoS Pathog 6, e1001210.

Kobayashi, S.D., DeLeo, F.R., 2013. *Staphylococcus aureus* protein A Promotes Immune Suppression. mBio 4.

Konig, U., Nyman, A.K., de Verdier, K., 2011. Prevalence of footrot in Swedish slaughter lambs. Acta Vet Scand 53, 27.

Kortt, A.A., Riffkin, M.C., Focareta, A., Stewart, D.J., 1993. Amino acid sequence of extracellular acidic protease V5 of *Dichelobacter nodosus*, the causative organism of ovine footrot. Biochem Mol Biol Int 29, 989-998.

Kuo, C.-H., Ochman, H., 2010. The Extinction Dynamics of Bacterial Pseudogenes. PLoS Genetics 6, e1001050.

La Fontaine, S., Egerton, J.R., Rood, J.I., 1993. Detection of *Dichelobacter nodosus* using species-specific oligonucleotides as PCR primers. Veterinary microbiology 35, 101-117.

La Ragione, R.M., Casula, G., Cutting, S.M., Woodward, M.J., 2001. Bacillus subtilis spores competitively exclude *Escherichia coli* O78:K80 in poultry. Vet Microbiol 79, 133-142.

Lane, D.J., 1991. 16S'23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, Edited by E. Stackebrandt & M. Goodfellow. Chichester : Wiley., 115-175.

Lavín, S., Ruiz-Bascarán, M., Marco, I., Abarca, M.L., Crespo, M.J., Franch, J. , 2004. Foot Infections Associated with *Arcanobacterium pyogenes* in free-living Fallow Deer (Dama dama) J Wildlife Dis 40, 607–611.

Lerat, E., Ochman, H., 2005. Recognizing the pseudogenes in bacterial genomes. Nucleic Acids Research 33, 3125-3132.

Lilley, G.G., Stewart, D.J., Kortt, A.A., 1992. Amino acid and DNA sequences of an extracellular basic protease of *Dichelobacter nodosus* show that it is a member of the subtilisin family of proteases. Eur J Biochem 210, 13-21.

Liu, D., Yong, W. K., 1995. Molecular basis for the virulence of *Dichelobacter nodosus*. Trends in Microbiology 3, 474-475.

Locatelli, A., Spor, A., Jolivet, C., Piveteau, P., Hartmann, A., 2013. Biotic and abiotic soil properties influence survival of *Listeria monocytogenes* in soil. PloS one 8, e75969.

Lund, M., Nordentoft, S., Pedersen, K., Madsen, M., 2004. Detection of *Campylobacter spp*. in chicken fecal samples by real-time PCR. Journal of clinical microbiology 42, 5125-5132.

M.R., P., 1991 The causative agent of foot-rot : description, pathogenicity, vaccination. Revue-de-Medecine-Veterinaire 6 453-462.

Ma, J., Ibekwe, A.M., Crowley, D.E., Yang, C.-H., 2014. Persistence of *Escherichia coli* O157 and non-O157 strains in agricultural soils. Science of the Total Environment 490, 822-829.

Mann, H.B., Whitney, D.R., 1947. On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other. 50-60.

Marsh, H., Tunnicliff, E.A. 1934. Experimental studies of foot-Rot in sheep (BuZZ. Mont. agric.Exp.Bta., No. 285.).

Mattick, J.S., Anderson, B.J., Mott, M.R., Egerton, J.R., 1984. Isolation and characterization of *Bacteroides nodosus* fimbriae: structural subunit and basal protein antigens. Journal of Bacteriology 160, 740-747.

Mavrogianni V.S, Menzies P.I, Fragkou I.A., 2011. Principles of mastitis treatment in sheep and goats. Veterinary Clinical North American Food Animal Practice 27:115-20.

Mohler, J.R., Washburn, H.J. 1905. Foot-rot of sheep: its nature, cause and treatment. Government Printing Office. Washington, DC, USA.

Moore, L.J., Wassink, G.J., Green, L.E., Grogono-Thomas, R., 2005a. The detection and characterisation of *Dichelobacter nodosus* from cases of ovine footrot in England and Wales. Vet Microbiol 108, 57-67.

Moore, L.J., Wassink, G. J., Green, L. E., Grogono-Thomas, R., 2005. The detection and characterisation of *Dichelobacter nodosus* from cases of ovine footrot in England and Wales. Veterinary microbiology 108, 57-67.

Moore, L.J., Woodward, M.J., Grogono-Thomas, R., 2005b. The occurrence of treponemes in contagious ovine digital dermatitis and the characterisation of associated *Dichelobacter nodosus*. Veterinary microbiology 111, 199-209.

Moran, N.A., 2002. Microbial Minimalism. Cell 108, 583-586.

Moynihan, E.L., Richards, K.G., Brennan, F.P., Tyrrel, S.F., Ritz, K., 2015. Enteropathogen survival in soil from different land-uses is predominantly regulated by microbial community composition. Applied Soil Ecology 89, 76-84.

Muzafar, M., Calvo-Bado, L.A., Green, L.E., Smith, E.M., Russell, C.L., Grogono-Thomas, R., Wellington, E.M.H., 2015a. The role of the environment in transmission of *Dichelobacter nodosus* between ewes and their lambs. Veterinary microbiology 179, 53-59.

Muzafar, M., Green, L.E., Calvo-Bado, L.A., Tichauer, E., King H., James P., Wellington, E.M.H., 2015b. Survival of ovine footrot pathogen *Dichelobacter nodosus* in different soils. Anaerobe 38, 81-87.

Myers, G.S., Parker, D., Al-Hasani, K., Kennan, R.M., Seemann, T., Ren, Q., Badger, J.H., Selengut, J.D., Deboy, R.T., Tettelin, H., Boyce, J.D., McCarl, V.P., Han, X., Nelson, W.C., Madupu, R., Mohamoud, Y., Holley, T., Fedorova, N., Khouri, H., Bottomley, S.P., Whittington, R.J., Adler, B., Songer, J.G., Rood, J.I., Paulsen, I.T., 2007. Genome sequence and identification of candidate vaccine antigens from the animal pathogen *Dichelobacter nodosus*. Nature biotechnology 25, 569-575.

Naylor, R.D., Martin, P.K., Jones, J.R., Burnell, M.C., 1998. Isolation of spirochaetes from an incident of severe virulent ovine footrot. The Veterinary record 143, 690-691.

Nebbia P, Robino P, Zoppi S., 2006. Detection and excretion pattern of *Mycobacterium avium* subspecies paratuberculosis in milk of asymptomatic sheep and goats by Nested-PCR. Small Ruminant Research 66:116-120.

Nei, M.a.K., S. , 2000. Molecular EŠolution and Phylogenetics. Oxford University Press,New York.

Niemann, S., Richter, E., Rüsch-Gerdes, S., 1999. Stability of *Mycobacterium tuberculosis* IS6110 Restriction Fragment Length Polymorphism Patterns and Spoligotypes Determined by Analyzing Serial Isolates from Patients with Drug-Resistant Tuberculosis. J Clin Microbiol 37, 409-412.

Nieuwhof, G.J., Bishop, S.C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Animal Science 81, 23-29.

Nocker, A., Mazza, A., Masson, L., Camper, A.K., Brousseau, R., 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. Journal of microbiological methods 76, 253-261.

Ochiai, S., Ishiko, H., Yasuda, M., Deguchi, T., 2008a. Rapid Detection of the Mosaic Structure of the *Neisseria gonorrhoeae* penA Gene, Which Is Associated with Decreased Susceptibilities to Oral Cephalosporins. Journal of clinical microbiology 46, 1804-1810.

Ochiai, S., Ishiko, H., Yasuda, M., Deguchi, T., 2008b. Rapid Detection of the Mosaic Structure of the Neisseria gonorrhoeae penA Gene, Which Is Associated with Decreased Susceptibilities to Oral Cephalosporins. J Clin Microbiol 46, 1804-1810.

Olson, M.E., Gard, M.S., Gradin, J., Morck, D.W., 1998. Serological classification and virulence determination of *Dichelobacter nodosus* isolated from Alberta and British Columbia sheep. Can J Vet Res 62, 33-37.

Palmer, M.A., 1993. A gelatin test to detect activity and stability of proteases produced by *Dichelobacter (Bacteroides) nodosus*. Veterinary microbiology 36, 113-122.

Pappas G, Papadimitriou P, Christou L., 2006. Future trends in human brucellosis treatment. Expert Opinion Investigating Drugs 15:1141-9.

Parajuli, B., Goddard, P.J., 1989. A comparison of the efficacy of footbaths containing formalin or zinc sulphate in treating ovine foot-rot under field conditions. British Veterinary Journal 145, 467-472.

Parker, D., Kennan, R.M., Myers, G.S., Paulsen, I.T., Songer, J.G., Rood, J.I., 2006. Regulation of type IV fimbrial biogenesis in *Dichelobacter nodosus*. J Bacteriol 188, 4801-4811.

Piriz Duran, S., Cuenca Valera, R., Valle Manzano, J., Vadillo Machota, S., 1990. Isolation and identification of anaerobic bacteria from ovine foot rot in Spain. Res Vet Sci 49, 245-247.

Piriz, S., Hurtado, M.A., Valle, J., Mateos, E.M., Martin-Palomino, P., Vadillo, S., 1996. Bacteriological study of footrot in pigs: a preliminary note. The Veterinary record 139, 17-19.

Pitman, D.R., Palmer, M.A., Depiazzi, L.J., 1994. The laboratory culture of *Dichelobacter nodosus* in a footrot eradication program. Australian veterinary journal 71, 109-112.

Raadsma, H.W., Egerton, J.R., 2013. A review of footrot in sheep: Aetiology, risk factors and control methods. Livestock Science 156, 106-114.

Raadsma, H.W., O'Meara, T.J., Egerton, J.R., Lehrbach, P.R., Schwartzkoff, C.L., 1994. Protective antibody titres and antigenic competition in multivalent *Dichelobacter nodosus* fimbrial vaccines using characterised rDNA antigens. Vet Immunol Immunopathol 40, 253-274.

Rasbash, J., Charlton, C., Browne , W.J., Healy, M., Cameron, B., 2009. MLwiN Version 2.1. Centre for Multilevel Modelling, University of Bristol.

Rattray, E.A.S., Prosser, J.I., Glover, L.A., Killham, K., 1992. Matric potential in relation to survival and activity of a genetically modified microbial inoculum in soil Soil Biol. Biochem. 24, 421-425.

Richards, R.B., Depiazzi, L.J., Edwards, J.R., Wilkinson, F.C., 1980. Isolation and characterisation of *Bacteroides nodosus* from foot lesions of cattle in western australia. Australian veterinary journal 56, 517-521.

Riffkin, M.C., Focareta, A., Edwards, R.D., Stewart, D.J., Kortt, A.A., 1993. Cloning, sequence and expression of the gene (aprV5) encoding extracellular serine acidic protease V5 from *Dichelobacter nodosus*. Gene 137, 259-264.

Riffkin, M.C., Wang, L.F., Kortt, A.A., Stewart, D.J., 1995. A single amino-acid change between the antigenically different extracellular serine proteases V2 and B2 from *Dichelobacter nodosus*. Gene 167, 279-283.

Roberts, D.S., Egerton, J.R., 1969. The aetiology and pathogenesis of ovine foot-rot. II. The pathogenic association of *Fusiformis nodosus* and *F. necrophorus*. J Comp Pathol 79, 217-227.

Roberts, D.S., Graham, N.P.H., Egerton, J.R., Parsonson, I.M., 1968. Infective bulbar necrosis (heel-abscess) of sheep, a mixed infection with *Fusiformis necrophorus* and *Corynebacterium pyogenes*. Journal of Comparative Pathology 78, 1-IN2.

Rollins, D.M., Colwell, R.R., 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Applied and Environmental Microbiology 52, 531-538.

Romero, D., Palacios, R., 1997. Gene amplification and genomic plasticity in prokaryotes. Annu Rev Genet 31, 91-111.

Rood, J.I., Howarth, P.A., Haring, V., Billington, S.J., Yong, W.K., Liu, D., Palmer, M.A., Pitman, D.R., Links, I., Stewart, D.J., Vaughan, J.A., 1996. Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of *Dichelobacter nodosus*. Veterinary microbiology 52, 127-141.

Rood, J.I., Stewart, D.J., Vaughan, J.A. & Dewhirst, F.E. , 2005. Bergey's Manual of Systematic Bacteriology Vol. 2: The Proteobacteria; Part B: The Gammaproteobacteria Edn. 2 (Brenner, D.J., Kreig, N.R. & Staley, J.T., eds.) Springer, New york, 124-129 pp.

Russell, C.L., Smith, E.M., Calvo-Bado, L.A., Green, L.E., Wellington, E.M., Medley, G.F., Moore, L.J., Grogono-Thomas, R., 2014. Multiple locus VNTR analysis highlights that geographical clustering and distribution of *Dichelobacter nodosus*, the causal agent of footrot in sheep, correlates with inter-country

movements. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 22, 273-279.

Sackett, D.M., Abbott, K. A., Jephcott, S, Barber, M. 2006. Assessing the economic cost of endemic disease on the profitability of Australian beef cattle and sheep. In: Meat and Livestock Australia, , Sydney.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406-425.

Schwartzkoff, C.L., Egerton, J.R., Stewart, D.J., Lehrbach, P.R., Elleman, T.C., Hoyne, P.A., 1993. The effects of antigenic competition on the efficacy of multivalent footrot vaccines. Australian veterinary journal 70, 123-126.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., Ishikawa, H., 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature 407, 81-86.

Short, J.A., Thorley, C.M., Walker, P.D., 1976. An Electron Microscope Study of *Bacteroides nodusus*: Ultrastructure of Organisms from Primary Isolates and Different Colony Types. Journal of Applied Bacteriology 40, 311-315.

Skerman, T.M., 1975. Determination of some in vitro growth requirements of *Bacteroides nodosus*. J Gen Microbiol 87, 107-119.

Smith, E.M., Green, O.D., Calvo-Bado, L.A., Witcomb, L.A., Grogono-Thomas, R., Russell, C.L., Brown, J.C., Medley, G.F., KilBride, A.L., Wellington, E.M., Green, L.E., 2014. Dynamics and impact of footrot and climate on hoof horn length in 50 ewes from one farm over a period of 10 months. Veterinary journal 201, 295-301.

Smith, J.M. 1996. Population genetics: an introduction. In: Neidhardt F C, Curtiss III R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M, Umbarger H E, editors. *Escherichia coli* and *Salmonella*: cellular and molecular biology. In: American Society of Microbiology, Washington, D.C., 2685–2690.

Smith, J.M., Dowson, C.G., Spratt, B.G., 1991. Localized sex in bacteria. Nature 349, 29-31.

Solovyev, V., Salamov, A. , 2011. Automatic Annotation of Microbial Genomes and Metagenomic Sequences. In Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies. Nova Science Publishers, 61-78 pp.

Srinivasan, S., Liu, C., Mitchell, C.M., Fiedler, T.L., Thomas, K.K., Agnew, K.J., Marrazzo, J.M., Fredricks, D.N., 2010. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. PloS one 5, e10197.

Stäuble, A., Steiner, A., Normand, L., Kuhnert, P., Frey, J., 2014. Molecular genetic analysis of Dichelobacter nodosus proteases AprV2/B2, AprV5/B5 and BprV/B in clinical material from European sheep flocks. Veterinary microbiology 168, 177-184.

Stewart, D., 1979. The role of elastase in differentiation of *Bacteroides nodosus* infections in sheep and cattle. Research in Veterinary Science 27, 99-105.

Stewart, D.J., 1973. An electron microscopic study of *Fusiformis nodosus*. Res Vet Sci 14, 132-134.

Stewart, D.J., 1989. Footrot of Sheep. In: J. R. Egerton, W. K. Yong and G. G. Riffin, eds. Footrot and Foot Abscess of Ruminants. CRC Press, Boca Raton, Florida, USA,.

Stewart, D.J., Clark, B.L., Jarrett, R.G., 1984. Differences between strains of *Bacteroides nodosus* in their effects on the severity of foot-rot, bodyweight and wool growth in Merino sheep. Australian veterinary journal 61, 348-352.

Stoltz, Y., 2013. Investigation into the expression and binding properties of the polymorphic protein Pgr, a virulence determinant of causal agent of ovine foot disease. University of Bielefeld, Germany,

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30, 2725-2729.

Thomas, J.H., 1958. A simple medium for the isolation and cultivation of *Fusiformis nodosus*. Australian veterinary journal 34, 411.

Thomas, J.H., 1962. The differential diagnosis of footrot in sheep. Australian veterinary journal 38, 366-367.

Thorley, C.M., 1976. A Simplified Method for the Isolation of *Bacteroides nodusus* from Ovine Foot-rot and Studies on its Colony Morphology and Serology. Journal of Applied Bacteriology 40, 301-309.

van der Woude, M.W., Bäumler, A.J., 2004. Phase and Antigenic Variation in Bacteria. Clinical Microbiology Reviews 17, 581-611.

van Elsas, J.D., Semenov, A.V., Costa, R., Trevors, J.T., 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. The ISME journal 5, 173-183.

Verstrepen, K.J., Jansen, A., Lewitter, F., Fink, G.R., 2005. Intragenic tandem repeats generate functional variability. Nat Genet 37, 986-990.

Walker, P.D., Short, J., Thomson, R. O., Roberts, D. S., 1973. The Fine Structure of *Fusiformis nodosus* with Special Reference to the Location of Antigens Associated with Immunogenicity. Microbiology 77, 351-361.

Walker, R.L., Read, D.H., Loretz, K.J., Nordhausen, R.W., 1995. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Veterinary microbiology 47, 343-355.

Wang, H., Ibekwe, A.M., Ma, J., Wu, L., Lou, J., Wu, Z., Liu, R., Xu, J., Yates, S.R., 2014a. A glimpse of *Escherichia coli* O157:H7 survival in soils from eastern China. Science of The Total Environment 476-477, 49-56.

Wang, H., Zhang, T., Wei, G., Wu, L., Wu, J., Xu, J., 2014b. Survival of *Escherichia coli* O157:H7 in soils under different land use types. Environ Sci Pollut Res Int 21, 518-524.

Wani, S.A., Samanta, I., 2006. Current understanding of the aetiology and laboratory diagnosis of footrot. Vet J 171, 421-428.

Wani, S.A., Samanta, I., Kawoosa, S., 2007. Isolation and characterization of *Dichelobacter nodosus* from ovine and caprine footrot in Kashmir, India. Res Vet Sci 83, 141-144.

Warren, R.M., van der Spuy, G.D., Richardson, M., Beyers, N., Borgdorff, M.W., Behr, M.A., van Helden, P.D., 2002. Calculation of the Stability of the IS6110 Banding Pattern in Patients with Persistent *Mycobacterium* tuberculosis Disease. J Clin Microbiol 40, 1705-1708.

Wassink, G.J., George, T.R., Kaler, J., Green, L.E., 2010a. Footrot and interdigital dermatitis in sheep: farmer satisfaction with current management, their ideal management and sources used to adopt new strategies. Prev Vet Med 96, 65-73.

Wassink, G.J., Green, L. E., Moore, L. J., Grogono-Thomas, R., 2006. Footrot and interdigital dermatitis in sheep. Veterinary Record 158 72.

Wassink, G.J., Grogono-Thomas, R., Moore, L.J., Green, L.E., 2003. Risk factors associated with the prevalence of footrot in sheep from 1999 to 2000. The Veterinary record 152, 351-358.

Wassink, G.J., Grogono-Thomas, R., Moore, L.J., Green, L.E., 2004. Risk factors associated with the prevalence of interdigital dermatitis in sheep from 1999 to 2000. The Veterinary record 154, 551-555.

Wassink, G.J., King, E.M., Grogono-Thomas, R., Brown, J.C., Moore, L.J., Green, L.E., 2010b. A within farm clinical trial to compare two treatments (parenteral antibacterials and hoof trimming) for sheep lame with footrot. Prev Vet Med 96, 93-103.

Wassink, G.J., Moore, L.J., Grogono-Thomas, R., Green, L.E., 2005. Footrot and interdigital dermatitis in sheep: farmers' practices, opinions and attitudes. The Veterinary record 157, 761-765.

Waters W.R, Palmer M.V, Buddle B.M., 2012. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine 30:2611-22.

Whittington, R.J., 1995. Observations on the indirect transmission of virulent ovine footrot in sheep yards and its spread in sheep on unimproved pasture. Australian veterinary journal 72, 132-134.

Whittington, R.J., Nicholls, P.J., 1995. Effects of the severity and duration of lesions on the primary and anamnestic humoral responses of sheep to *Dichelobacter nodosus* and observations of natural resistance to footrot. Res Vet Sci 59, 128-135.

Whittle, G., Katz, M.E., Clayton, E.H., Cheetham, B.F., 2000. Identification and Characterization of a Native *Dichelobacter nodosus* Plasmid, pDN1. Plasmid 43, 230-234.

Windsor P.A., 2015. Paratuberculosis in sheep and goats. Veterinary Microbiology 181:161-9.

Winter, A.C., 2008. Lameness in sheep. Small Ruminant Research 76, 149-153.

Witcomb, L.A., 2012. University of Warwick. The in situ analysis of the microbial community associated with footrot of sheep. Ph.D. thesis.

Witcomb, L.A., Green, L.E., Kaler, J., Ul-Hassan, A., Calvo-Bado, L.A., Medley, G.F., Grogono-Thomas, R., Wellington, E.M., 2014. A longitudinal study of the role of *Dichelobacter nodosus and Fusobacterium necrophorum* load in initiation and severity of footrot in sheep. Prev Vet Med 115, 48-55.

Young, J.S., Gormley, E., Wellington, E.M., 2005. Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. Appl Environ Microbiol 71, 1946-1952.

Zakaria, Z., Radu, S., Sheikh-Omar, A.R., Mutalib, A.R., Joseph, P.G., Rusul, G., 1998. Molecular analysis of *Dichelobacter nodosus* isolated from footrot in sheep in Malaysia. Veterinary microbiology 62, 243-250.

Zhou, H., Hickford, J.G., 2000. Extensive diversity in New Zealand *Dichelobacter nodosus* strains from infected sheep and goats. Veterinary microbiology 71, 113-123.

Zhou, H., Lottner, S., Ganter, M., Hickford, J.G.H., 2010. Identification of two new *Dichelobacter nodosus* strains in Germany. The Veterinary Journal 184, 115-117.

APPENDIX

Appendix 1:

Bacterial strains used in this thesis, type of the sample and their source

Bacterial Strain	Type of the sample	Source
*Dichelobacter nodosus*_Strain VCS1703A	Isolate and DNA	Monash University, Melbourne
*Dichelobacter nodosus*_Strain C305	Isolate and DNA	Monash University, Melbourne
*Dichelobacter nodosus*_Strain BS4	Isolate and DNA	University of Bristol, England
Escherichia coli	DNA	Nikki Buller, Perth, Australia
Fusobacterium necrophorum	DNA	Nikki Buller, Perth, Australia
Lactococcus garvieae	DNA	Nikki Buller, Perth, Australia
Leishmania donowani	DNA	Nikki Buller, Perth, Australia
Mycobacterium intracellulare	DNA	Nikki Buller, Perth, Australia
Prevotella oralis	DNA	Nikki Buller, Perth, Australia
Propionibacterium acnes	DNA	Nikki Buller, Perth, Australia
Staphylococcus aureus	DNA	Nikki Buller, Perth, Australia
Staphylococcus intermedius	DNA	Nikki Buller, Perth, Australia
Staphylococcus pseudintermedius	DNA	Muriel Breteau, Warwick University, UK
Synechococcus	DNA	Branco, Warwick University, UK
BCG Tuberculosis	DNA	David Porter, Warwick University, UK
DNA isolated from a slaughter house dust from Tanzania	DNA	David Porter, Warwick University, UK

Appendix 2:

Sequences submitted to GenBank.

***pgr* sequences containing the variable number of tandem repeats in the R1 region.**

>MM_Clone1 (Organism = *Dichelobacter nodosus)* Tandem repeats 3

gcttttgctgtgggtgcggcagctgtcggtggtttggctattaagacgcacggcgaagacgataaacccaaatcccacga
cgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaaccg
ggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagttt
aacaagcatggtgcaggt

Protein Translation:
afavgaaavgglaikthgeddkpkshdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgentelikpk
lefnkhgag

>MM_Clone2(Organism = *Dichelobacter nodosus)* Tandem repeats 4

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacgat
gagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaatcag
gtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaat
aaaaccaaaattagagtttaacaagcatggtgcaggt

Protein Translation:
afavgaaavgglaikthgeddkpkphdeiykdkiddapeegapkmppesgeksgsgekpgsgekpgsgekpgsgen
telikpklefnkhgag

>MM_Clone3(Organism = *Dichelobacter nodosus)* Tandem repeats 5

gcttttgctgtgggtgcggcagctgttggtggttcggctattaagacgcacggcgaagacgataaacccaaatcccacga
cgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaacca
ggtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagt
ggtgagaacactgaattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein translation:
Afavgaaavggsaikthgeddkpkshdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefnkhgag

>MM_Clone4 (Organism = *Dichelobacter nodosus)* Tandem repeats 6

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacgacgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein translation:
afavgaaavgglaikthgeddkpkphdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefnkhgag

>MM_Clone5 (Organism = *Dichelobacter nodosus)* Tandem repeats 11

gcttttgctgtgggtgcggcagccgttggtggtttggctattaagacgcacggcgaagacgataaacccaaatcccacgacgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagcggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein Translation:
afavgaaavgglaikthgeddkpkshdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefnkhgag

>MM_Clone6 (Organism = *Dichelobacter nodosus)* Tandem repeats 12

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacgacgagatttataaagataagactgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein translation:
afavgaaavgglaikthgeddkpkphdeiykdktddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefnkhgag

>MM_Clone7(Organism = *Dichelobacter nodosus)*Tandem repeats 13

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacga
cgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaacca
ggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtg
gtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccaggtagtggtgaaa
aaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactg
aattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein translation:
afavgaaavgglaikthgeddkpkphdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefnkhgag

MM_Clone8(Organism = *Dichelobacter nodosus)* Tandem repeats 15

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacga
cgagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaacca
ggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtg
gtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaa
aaccgggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccgg
gtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaacaagcatggtgcaggt

Protein translation:
afavgaaavgglaikthgeddkpkphdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgentelikpklefn
khgag

>MM_Clone9(Organism = *Dichelobacter nodosus)* Tandem repeats 16

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacga
cgagatttataaagataagattgatgatgctccagaagaaggggtgcctaagatgccgccagaaagtggtgaaaaaccag
gtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtgg
tgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaa
ccaggtagtggtgaaaaaccgggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggt
agtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaa
caagcatggtgcaggt

Protein translation:

afavgaaavgglaikthgeddkpkphdeiykdkiddapeegvpkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgenteli
kpklefnkhgag

>MM_Clone10 (Organism = *Dichelobacter nodosus)* Tandem repeats 20

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacacga
cgagatttataaagataagattgatgatgctccagaagaaggggtgcctaagatgccgccagaaagtggtgaaaaaccag
gtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtgg
tgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaa
ccaggtagtggtgaaaaaccgggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccgggt
agtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgaaaaaccaggtagtggt
gaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacactgaattaataaaaccaaaattagagtttaacaagca
tggtgcaggt

Protein translation:

afavgaaavgglaikthgeddkpkphdeiykdkiddapeegvpkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgs
gekpgsgekpgsgekpgsgentelikpklefnkhgag

>MM_Clone11_(Organism = *Dichelobacter nodosus)* Tandem repeats 21

gcttttgctgtgggtgcggcagctgttggtggtttggctatcaagacgcacggcgaagacgataaacctaaaccacatgac
gagatttataaagataagattgatgatgctccagaagaaggggcgcctaagatgccgccagaaagtggtgaaaaaccag
gtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtgg
tgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaa
ccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggtgaaaaaccaggt
agtggtgaaaaaccaggtagtggtgaaaaaccgggtagtggtgaaaaaccaggtagtggtgaaaaaccaggtagtggt
gaaaaaccgggtagtggtgaaaaaccgggtagtggtgaaaaaccgggtagtggtgagaacgctgaattaataaaaccaa
aattagagtttaacaagcatggtgcaggt

Protein translation:
afavgaaavgglaikthgeddkpkphdeiykdkiddapeegapkmppesgekpgsgekpgsgekpgsgekpgsgek
pgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgsgekpgs
gekpgsgekpgsgekpgsgekpgsgenaelikpklefnkhgag

Appendix 3:

Anaerobic cultivation of *Dichelobacter nodosus*

Anaerobic cabinet and growth and storage conditions	**Constituents**
Don Whitley III, *D*. *nodosus* was cultured on TASH plates and incubated at 37 ^{o}C for 4-5 days. The isolates to harvested in 20% glycerol to prepare the stocks for future use.	Anaerobic mix containing - Nitrogen 80%; Carbon dioxide 10%; Hydrogen 10%.

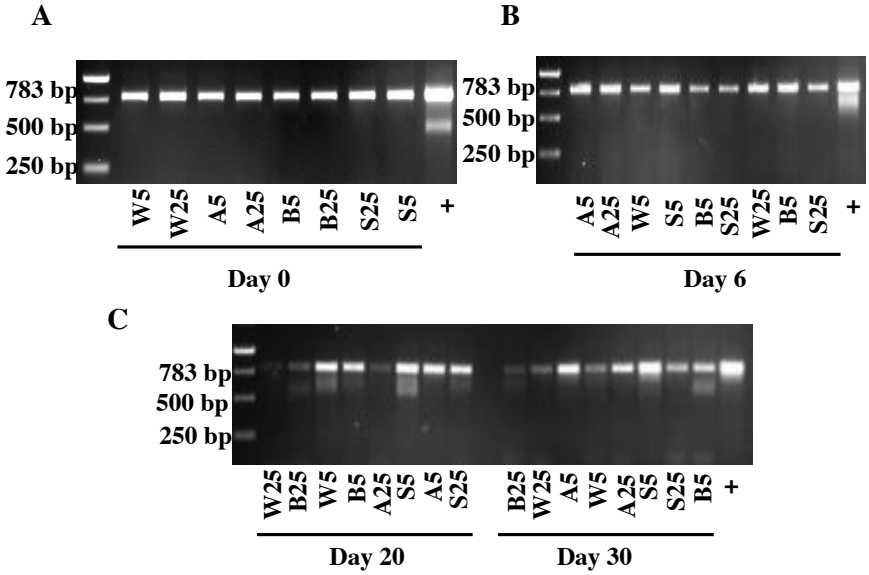
Appendix 4:

Concentration of the RNA extracted from the soil microcosms. The RNA concentration was measured using the Agilent Bioanalyser.

Sample	RNA Concentration (ng/ μl)
Day 1	71
Day 2	63
Day 3	59
Day 6	33
Day 10	29
Day 14	13
Day 21	10

Appendix 5:

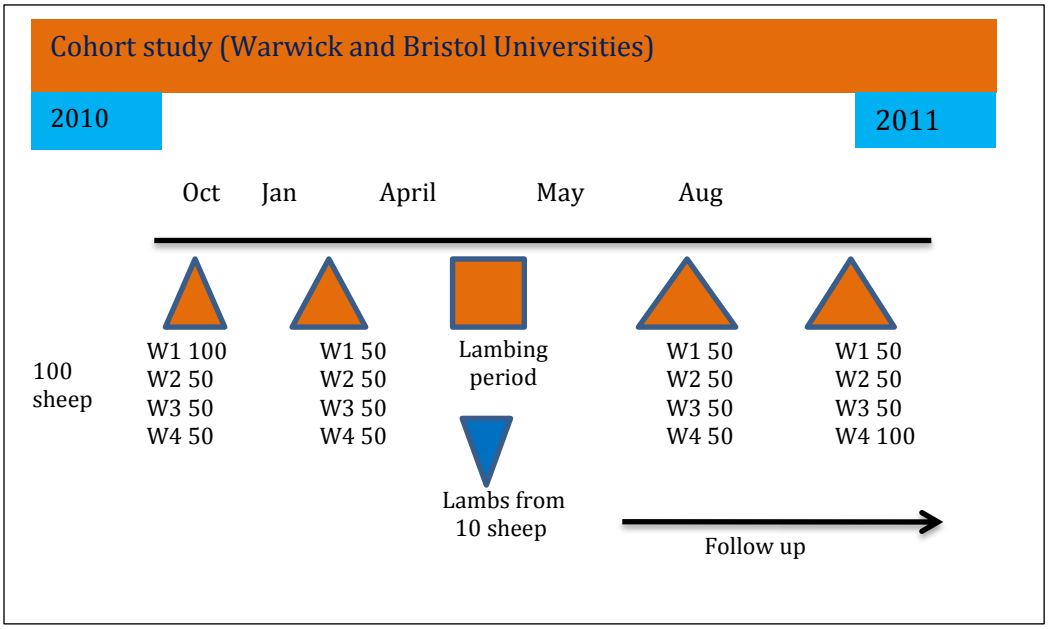
Persistence of *D. nososus* DNA in different soils.



Appendix 3: Persistence of *D. nodosus* DNA in all soil type confirmed by a specific 16S rRNA gene PCR. **(A, B, C)** Agarose gel showing DNA positive for *D. nodosus* in the samples at days 0, 6, 20 and 30 (W=Warwick; S=Stockton; A=Abanilla; B=Basilicata. 5= Temperature at 5 ^{o}C and 25=Temperature at 25 ^{o}C).

Appendix 6:

Layout of the cohort study



Appendix 7:

Amino acid sequence of PgrA protein used to predict the transmembrane localisation. The sequence in red is the N-terminus ASN 746 disordered region observed up to THR 776, followed by two beta pleated sheets, the sequence for the first beta pleated sheet is highlighted in green (A: SER859 to GLU 972, B) and the sequence for the second is highlighted in pink (GLY 984 to SER 1094) conjoined with a short linker highlighted in brown.

ASN746-
NTEVTASVTNASGSAAGEKTDVTVIALAPATVELDNIDGDLTTKGVPAGVD
DNARPSDWINNYHVKGDIKSADGSIVIVDTAGGRDSLIVDGDQVNSIVYLGN
DNDIEHVNNVSDSSIFAGKGNDAITIDGNVANSYISTGGSTTIGSIEGSDTINIG
GNVSGSTIDLGSGLPMPEQYAPVNPDKDDNVGHNAQLTVDGSFEHSTVHGS
QGVDMVEFNHVGDGAKVELGNSDDILVLSGTFDGSAQDVMLDGGAGSDTL
VIGGSGSNLSIASLKSFETINLDEGGHNLNMLDFDAFKAFMGDNTELYLNGD
NSNHIFMDKTGTWTRDEAPQDSGDYAPVDHDGYHLYTSKEGGYQL – 1101

Appendix 8:

Constituents of Luria Bertani (LB) Agar/Broth (1 litre).

Peptone	10 g
Yeast extract	05 g
Sodium hydroxide	10 g
Agar powder	10 g

The general laboratory reagents were procured from Sigma-Aldrich Ltd (Poole, Dorset – United Kingdom).